Technical Report on Mirror Bacteria: Feasibility and Risks

December, 2024

Abstract

This report describes the technical feasibility of creating mirror bacteria and the potentially serious and wide-ranging risks that they could pose to humans, other animals, plants, and the environment. It accompanies the *Science* Policy Forum article titled “Confronting risks of mirror life”, published in December 2024.

In a mirror bacterium, all of the chiral molecules of existing bacteria—proteins, nucleic acids, and metabolites—are replaced by their mirror images. Mirror bacteria could not evolve from existing life, but their creation will become increasingly feasible as science advances. Interactions between organisms often depend on chirality, and so interactions between natural organisms and mirror bacteria would be profoundly different from those between natural organisms. Most importantly, immune defenses and predation typically rely on interactions between chiral molecules that could often fail to detect or kill mirror bacteria due to their reversed chirality. It therefore appears plausible, even likely, that sufficiently robust mirror bacteria could spread through the environment unchecked by natural biological controls and act as dangerous opportunistic pathogens in an unprecedentedly wide range of other multicellular organisms, including humans.

This report draws on expertise from synthetic biology, immunology, ecology, and related fields to provide the first comprehensive assessment of the risks from mirror bacteria. It consists of eight chapters and starts with a general introduction, followed by an examination of the initial creation of mirror bacteria, their further engineering, as well as biosecurity and biosafety implications. The remaining five chapters cover risks to human health, medical countermeasures, risks to other animals, risks to plants, and the potential ecological consequences of their introduction into the environment.

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Review

Prior to release, feedback on scope, completeness, accuracy, and presentation of the analysis was solicited from scientific experts in each of the areas covered by the report. Experts were invited to comment on one or several chapters of the report. The authors of each chapter retained final editorial authority over the content and integration of expert feedback. Providing review comments does not imply endorsement of the report's overall findings or conclusions. Experts were given the option to be publicly acknowledged in this report. Reviewers who agreed to be acknowledged were (in alphabetical order):

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About this Report

This technical report analyzes the feasibility of creating mirror bacteria, evaluates the possible consequences of their release into the environment, and outlines some potential countermeasures.

Many authors of this report are co-authors of the article "Confronting risks of mirror life", published in *Science* in December 2024. Both that article and this technical report emerged from the activities of an international working group of scientific experts from a range of disciplines. This technical report informed the findings and recommendations of the *Science* article; while that article contains policy recommendations, this report focuses solely on technical analysis and risk assessment. The views expressed in this report reflect those of the authors and not necessarily those of their affiliated institutions.

Rationale for Public Release

Releasing this report inevitably draws attention to a potentially destructive scientific development. We do not believe that drawing attention to threats is always the best approach for mitigating them. However, in this instance we believe that public disclosure and open scientific discussion are necessary to mitigate the risks from mirror bacteria. We have two primary reasons to believe disclosure is necessary:

1) To prevent accidents and well-intentioned development

If no serious concerns are raised, the default course of well-intentioned scientific and technological development would likely result in the eventual creation of mirror bacteria. Creating mirror life has been a long-term aspiration of many academic investigators, and efforts toward this have been supported by multiple scientific funders.[1] While creating mirror bacteria is not yet possible or imminent, advances in enabling technologies are expected to make it achievable within the coming decades. It does not appear possible to develop these technologies safely (or deliberately choose to forgo them) without widespread awareness of the risks, as well as deliberate planning to mitigate them.

This concern is compounded by the possibility that mirror bacteria could accidentally cause irreversible harm even without intentional misuse. Without awareness of the threat, some of the most

[1] For instance, in 2019 the U.S. National Science Foundation awarded $4 million to a group of investigators seeking "to design, construct, and safely deploy synthetic mirror cells in which all of the key molecules—nucleic acids, proteins, carbohydrates, and lipids—exist in chiral states opposite to their natural forms" (National Science Foundation Award #1935120, #1935372). The National Natural Science Foundation of China has similarly supported work toward the creation of a mirror-image central dogma (National Natural Science Foundation of China grants 32050178 and 21925702), and the European Commission ERA-Net MirrorBio consortium, with similar goals, was supported by the German Federal Ministry of Education and Research (Bundesministerium für Bildung und Forschung grant 031A461).

dangerous modifications would likely be made for well-intentioned reasons, such as endowing mirror bacteria with the ability to metabolize D-glucose to allow growth in standard media.

2) To build guardrails that could reliably prevent misuse

There are currently substantial technical barriers to creating mirror bacteria. Success within a decade would require efforts akin to those of the Human Genome Project or other major scientific endeavors: a substantial number of skilled scientists collaborating for many years, with a large budget and unimpeded access to specialized goods and services. Without these resources, entities reckless enough to disregard the risks or intent upon misuse would have difficulty creating mirror bacteria on their own. Disclosure therefore greatly reduces the probability that well-intentioned funders and scientists would unwittingly aid such an effort while providing very little actionable information to those who may seek to cause harm in the near term.

Crucially, maintaining this high technical barrier in the longer term also appears achievable with a sustained effort. If well-intentioned scientists avoid developing certain critical components, such as methods relevant to assembling a mirror genome or key components of the mirror proteome, these challenges would continue to present significant barriers to malicious or reckless actors. Closely monitoring critical materials and reagents such as mirror nucleic acids would create additional obstacles. These protective measures could likely be implemented without impeding the vast majority of beneficial research, although decisions about regulatory boundaries would require broad discussion amongst the scientific community and other stakeholders, including policymakers and the public. Since ongoing advances will naturally erode technical barriers, disclosure is necessary in order to begin discussions while those barriers remain formidable.

Content Decisions

An understanding of both the major technical barriers and how they could be eroded by technological progress over the coming decades is necessary for grounded scientific deliberation and the development of effective guardrails. For this reason, Chapters 2 and 3 provide a high-level assessment of the potential pathways by which mirror bacteria might be created and then transformed into other species. They do not provide explicit, detailed technical instructions for the creation of a mirror bacterium (and given the immaturity of the precursor technologies, doing so would be impossible at this stage).

As a matter of prudence, our discussion of risks focuses on mirrored versions of existing bacteria, particularly those that are commonly studied and engineered, as well as traits that could arise naturally through mutation. We also discuss certain modifications to mirror bacteria that legitimate researchers would likely pursue if unaware of the risks described in this report. We intentionally do not discuss possible modifications that would serve only destructive purposes.[2]

[2] The closest the report comes to this is in Chapter 3, where we describe at a high level how a malicious or reckless actor could remove added safety mechanisms. We believe such analysis is essential for understanding which, if any, biocontainment strategies are promising.

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Summary

Many of the key molecules common to all living organisms are chiral: their mirror images cannot be superimposed upon one another. In nature, nearly all amino acids and sugars are predominantly present as only one of their two possible mirror-image forms. Synthetic mirror polypeptides and nucleic acids fold into perfect mirror images of their natural counterparts and interact identically with the mirror images of their natural substrates. The same principle applies to other biological structures. Scaling this logic, a mirror cell in which all molecules were replaced with their mirror-images would function identically to a natural-chirality cell, but with everything precisely mirrored.

Scientists have long considered the possibility of creating mirror cells, including mirror bacteria. In isolation, they would behave identically to natural-chirality bacteria—mirror bacteria would grow under the same physical conditions, and would grow at the same rates on achiral nutrients. But because natural life is chiral, interactions between natural organisms and mirror bacteria would be profoundly altered. This report describes the technical feasibility of creating mirror bacteria, and the potentially serious risks that mirror life could pose to humans, other animals, plants, and the environment.

Robust mirror bacteria could be developed in the coming years and would be vulnerable to accidental or deliberate misuse

It is not yet possible to create a living cell from non-living precursors. Despite major technical challenges, it appears plausible that this feat could be achieved within as little as a decade given sufficient resources. Once methods of creating natural-chirality cells have been established, a similar approach could be used to create mirror cells from mirror components. A small group of researchers and funders have begun to explicitly work toward the creation of mirror life, with a focus on a "bottom-up" approach, in which a mirror genome as well as transcription and translation machinery are used to build other mirror cellular components. Modern methods for protein synthesis are in principle sufficient to synthesize most of the mirror proteins that would be needed to construct a mirror cell, though currently at prohibitive costs. Mirror-image enzymes have recently been used to construct kilobase-length mirror RNA and DNA, and research is progressing toward building a mirror ribosome.

Other pathways to mirror bacteria appear possible. For example, a "top-down" approach, in which a natural-chirality bacterium is stepwise converted into a mirror bacterium, may become technically feasible as translational engineering advances.

The first mirror bacterium would likely be a fragile microbe exhibiting metabolic defects, which would limit its growth and durability outside the laboratory. Once created, however, mirror bacteria could be readily engineered to become more robust by using standard techniques to deliver mirror versions of existing bacterial genes. This could confer new capabilities or even transform them into

mirrored versions of robust existing bacteria. Adding mirrored versions of known metabolic pathways would allow mirror bacteria to utilize natural-chirality glucose and other common sugars.

Biocontainment measures could ensure that mirror bacteria are incapable of survival outside of a lab. However, these measures would be vulnerable to human error or deliberate misuse. Achieving robust biocontainment that could not be removed by a moderately skilled actor with access to mirror DNA does not appear feasible.

Mirror bacteria could evade many aspects of human immunity and potentially cause life-threatening infection

Most immunological mechanisms rely on precise stereospecific interactions between host and pathogen macromolecules. The mirror-image macromolecules of mirror bacteria would likely not properly bind to host receptors, enzymes, or other host effectors, as they would have the "wrong" chirality. This could grant mirror bacteria a degree of intrinsic immune evasion well beyond any known natural pathogen.

The immune system could be compromised in three key ways. First, the innate immune response relies upon initial detection of conserved microbial biomolecules, such as bacterial lipopolysaccharides and peptidoglycans, by host pattern recognition receptors. Because these molecules are almost exclusively chiral, immune recognition of mirror bacteria could be substantially impaired. Second, many innate immune mechanisms of pathogen control could be directly compromised; for example, phagocytosis, antimicrobial enzymes, and several complement system pathways rely on stereospecific protein interactions. Finally, mirror proteins would resist degradation and other stereospecific mechanisms necessary for antigen processing and presentation by innate immune cells, which would impair the activation of adaptive T and B immune cells and antibody production.

Human exposure to intrinsically immune-evasive mirror organisms may lead to infection and possibly death. While mirror bacteria would lack functional virulence factors that facilitate invasion, transient bacteremias caused by environmental bacteria are common, suggesting that exposure to mirror bacteria through inhalation, ingestion, microabrasions, or wounds could result in passive translocation across epithelial barriers.

Once inside, mirror versions of common bacteria such as *Escherichia coli* would be able to grow on achiral nutrients such as glycerol and, with suitable engineering, on glucose and other common chiral nutrients. Impaired immunity would likely permit extensive replication within the bloodstream. The exact clinical presentation of a mirror bacterial infection is unclear, but absent an effective immune response, a lethal outcome appears highly plausible.

Treatment options would be limited. Most antibiotics interact stereospecifically with their microbial targets, so existing stocks would be restricted to a few achiral or racemic antibiotics. As an emerging infectious disease, there would be no pre-existing vaccine. It should be possible to develop novel antimirror compounds and conjugate vaccines; nevertheless, as with a new pandemic, the practical challenges to developing such measures quickly and at scale would be considerable.

Mirror bacteria could potentially infect many other animals and plants and colonize some external environments, causing irreversible ecological disruption

Other multicellular organisms may be similarly vulnerable to mirror bacterial infection. Vertebrates share broadly similar immune systems and would likely suffer from equivalent defects to humans. Invertebrate immune systems are more variable and less thoroughly characterized, but may be similarly ineffective. For example, the model insect *Drosophila melanogaster* relies on peptidoglycan recognition to initiate antibacterial immune mechanisms, and so is unlikely to recognize mirror bacteria. The model nematode *Caenorhabditis elegans* does not appear to rely on the recognition of pathogen-associated molecules, but instead on pathogen avoidance and the recognition of host damage; the latter may or may not permit nematodes to survive a mirror bacterial infection. The consumption of tissues from an infected organism by a predator or scavenger could lead to infection, causing mirror bacteria to spread through the food web.

Plant immune systems also rely on the detection of conserved pathogen biomolecules. Almost all known immune receptors in plants recognize chiral ligands, and thus would be unlikely to recognize mirror bacteria. Although mirror bacteria would not by default possess specific adaptations to invade and colonize plants, both plant roots and leaf stomata could allow for passive translocation of mirror bacteria from the environment. Whether mirror bacteria could spread through vasculature is unclear and may vary for different types of plants, but if systemic infections did occur they might be fatal. Key crops could probably be engineered to be resistant, but protecting wild plants and their associated ecosystems would be infeasible.

In addition to functioning as a dangerous “accidental pathogen” to a wide range of natural-chirality species, mirror bacteria could persist within and potentially colonize external environments. Unlike their natural chirality counterparts, mirror bacteria would be completely resistant to all bacteriophages, partially evasive of and largely indigestible to predators, and largely resistant to antimicrobial compounds released by competing microbial species. These potentially decisive competitive advantages could allow sufficiently robust mirror bacteria to successfully invade many ecological niches despite lacking specific adaptations for them. Because predators would not be able to digest most mirror macromolecules, a growing mirror bacterial population would not be controlled by any commensurate increase in predation, which could allow populations to reach high abundance.

Once released, invasive mirror bacteria could be spread rapidly by infected animals and humans, and by the transport of contaminated goods and produce. While a released mirror bacterium would likely have little pre-existing genetic variation and no ability for gene transfer with natural-chirality bacteria, evolutionary diversification and adaptation to expand the environmental and host range of mirror bacteria would ensue.

Mirror bacteria may directly drive vulnerable plant and animal species to extinction, and the loss of vulnerable “keystone species” could indirectly trigger severe ecological disruptions. Very large mirror bacterial populations, especially autotrophic mirror bacteria, may disrupt nutrient cycling in many ecosystems, and could impact the global carbon cycle. Ecological countermeasures such as the synthesis and release of mirror phages that target the invasive mirror bacteria could reduce their maximum population size, but would have little realistic prospect of averting these large and irreversible harms.

Chapter 1: Introduction

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All known life on Earth shares a common set of chiral molecules. Proteins, sugars, lipids, and nucleic acids can all physically exist in either of two mirror-image configurations, but living organisms overwhelmingly use only one of these two configurations. By convention, one member of the pair is designated to be the L, or "left-handed" form, while the other is designated the D, or "right-handed" form. Virtually all proteins are constructed solely from the 20 canonical amino acids, of which all but achiral glycine are L-amino acids (Figure 1.1). Their mirror images are D-amino acids, which play only marginal roles in existing life. The ribose in RNA is always in the form of D-ribose; its mirror image L-ribose is essentially absent from nature (Figure 1.2).

Pairs of molecules that are mirror images of each other are known as enantiomers, which have almost exactly mirrored physical and chemical properties (see Box 1.1). Their crystalline structures are mirror images of each other and melt at the same temperatures. They have identical solubilities in water and identical acid-base dissociation constants, but they rotate polarized light in opposite directions.

Proteins are large, chiral molecules. Just as a protein with a specific sequence of L-amino acids will fold into a specific configuration, a mirror-image protein with the same specific sequence of D-amino acids folds into a configuration precisely mirroring that of the natural-chirality protein (Figure 1.3). If a protein naturally binds to some small molecule, an exact mirror-image of that protein will bind equally well to the enantiomer of that small molecule. This exact mirroring extends to catalytic activity: mirror-image enzymes will catalyze reactions involving the enantiomers of the products and substrates of the natural enzyme (Figure 1.4). Despite being exactly as versatile as natural-chirality proteins, mirror proteins cannot be made by natural living organisms and do not exist in nature.

Other mirror-image macromolecules also precisely mirror natural-chirality macromolecules. Mirror DNA, made with L-deoxyribose rather than D-deoxyribose, forms an opposite-handed double helix

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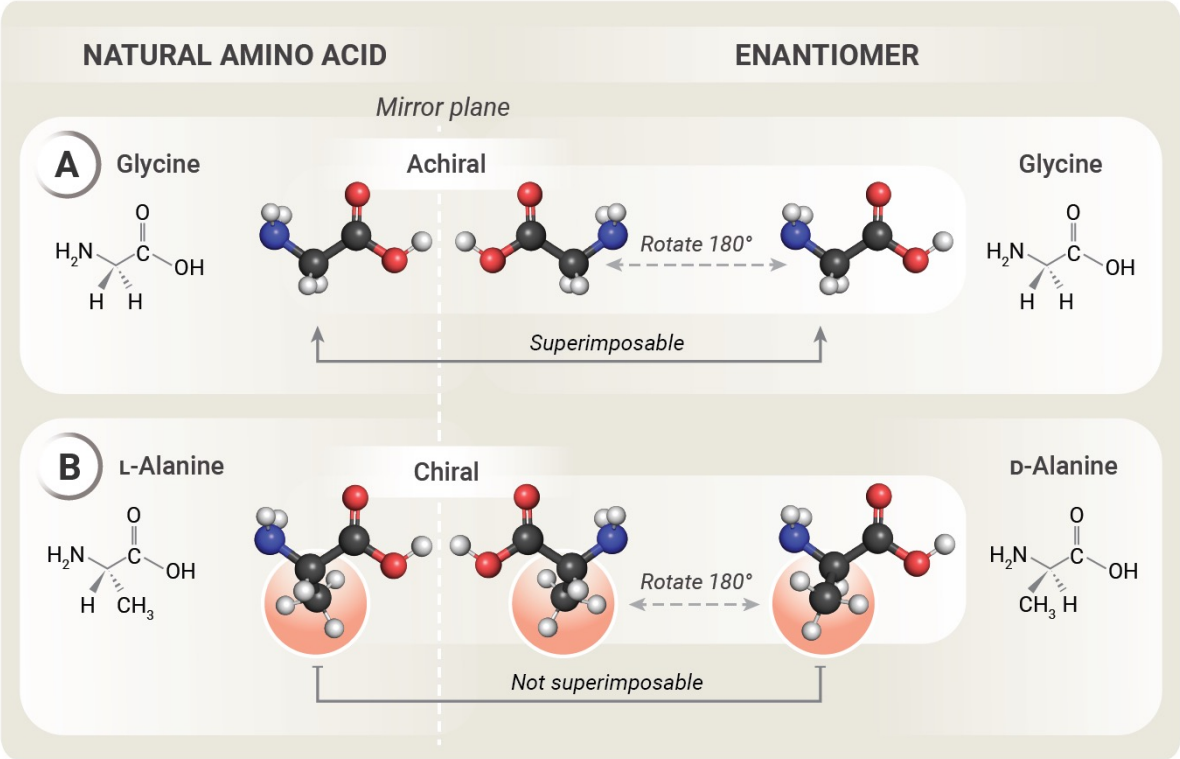

Figure 1.1: Chiral molecules have non-superimposable mirror images
A. The amino acid glycine is an achiral molecule, as it can be superimposed on its mirror image. **B.** All other canonical amino acids are chiral molecules, with two non-superimposable mirror images. Alanine is illustrated as an example. Even if D-alanine is rotated 180° so its amino and carboxyl groups are in the same orientation as L-alanine, as shown in the figure, the two cannot be superimposed because the methyl group in L-alanine is projecting into the page but the methyl group in D-alanine is projecting out of the page.

that precisely mirrors the double helix of natural-chirality DNA. Mirror nucleic acid polymerases could make new mirror DNA and mirror RNA from mirror nucleotides, and mirror ribosomes could synthesize mirror proteins from mirror amino acids using mirror mRNA templates (Figure 1.5).

Given the symmetry between enantiomers, it should in theory be possible to construct an organism entirely out of mirror biomolecules. Replacing every single molecule in a bacterium with its enantiomer would result in a "mirror bacterium" that would behave identically to a natural-chirality bacterium except for being mirrored. Such a mirror bacterium would natively consume "opposite" chirality nutrients relative to natural bacteria; for example, it would utilize L-glucose rather than D-glucose. But alone in an achiral medium, such as minimal media with achiral glycerol for a carbon source, a mirror bacterium should behave indistinguishably from its mirror-image natural-chirality bacterium.

It is well known that protein binding is stereospecific and therefore generally only one member of an enantiomeric pair is recognized by its binding partner. For example, the enantiomers of chiral drugs typically bind different receptors and consequently have different biological effects (Shen *et al.*, 2013; Smith, 2009). Most famously, thalidomide was originally approved as a treatment for morning

sickness, but it was manufactured as a racemic mixture of both enantiomers. One enantiomer acts as a mild sedative, but the other is harmful to dividing cells and can cause severe birth defects (Tokunaga *et al*., 2018). Similarly, enantiomers often have different odors because they bind to different olfactory receptors; one enantiomer of the oil carvone smells like caraway, while the other smells like spearmint (Bentley, 2006). Larger mirror macromolecules are also generally not recognized by natural enzymes. Proteases, for instance, are generally unable to cleave D-peptides (Miller *et al*., 1995), which therefore have much longer half-lives in the body, making them potentially useful therapeutics (Lander *et al*., 2023).

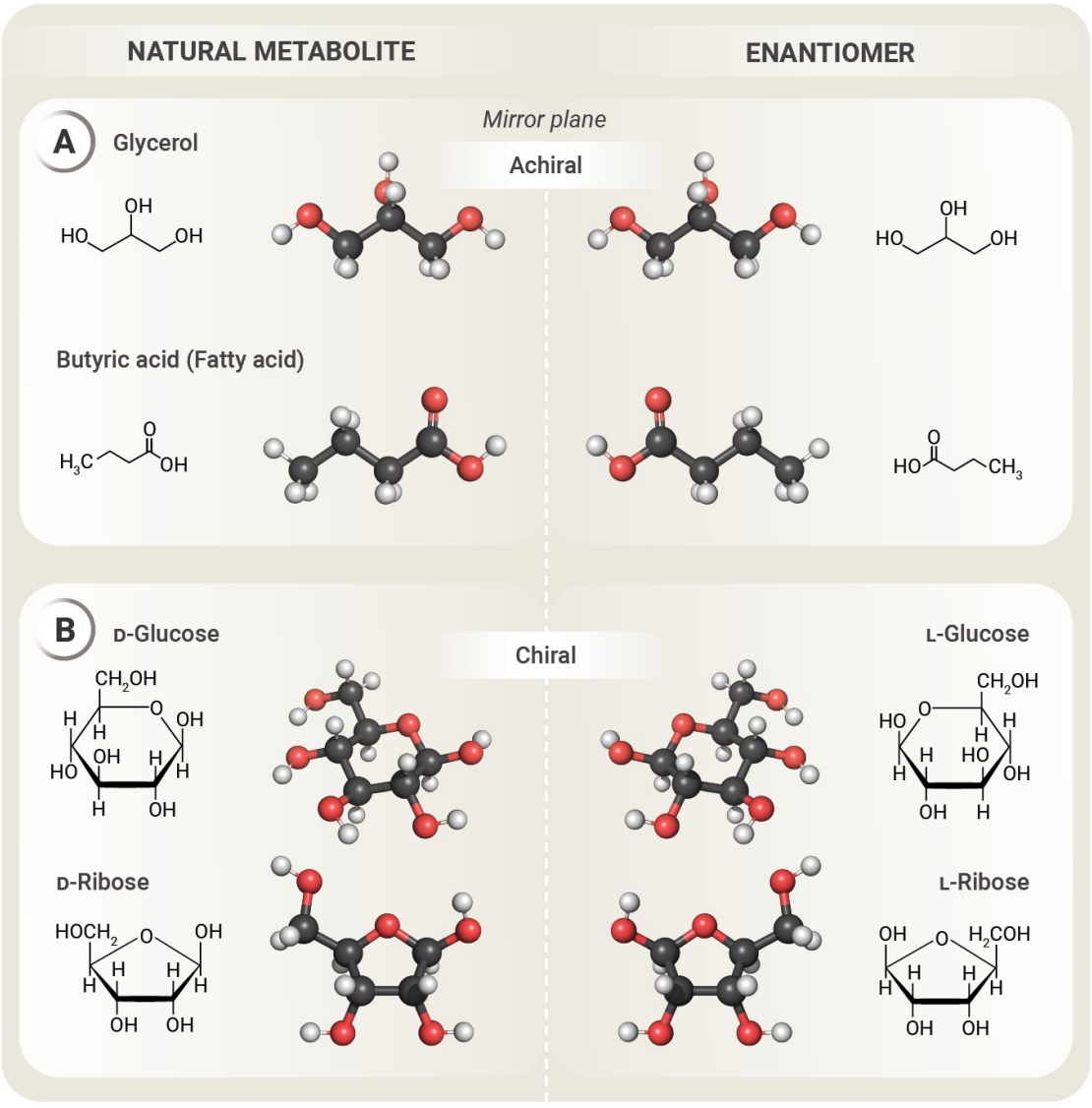

Figure 1.2: Living organisms are built from both chiral and achiral molecules
A. Glycerol and simple fatty acids are common in animals and plants; they are also achiral molecules that can be superimposed on their mirror images. **B.** Sugars are among the most abundant biological molecules on Earth, and are almost always chiral. For example, both glucose and ribose have two enantiomers (L- and D-) which are non-superimposable mirror images of each other. Living organisms use only one of the available enantiomers of chiral molecules: all RNA in all organisms on Earth is composed of D-ribose, while L-ribose is essentially absent from nature.

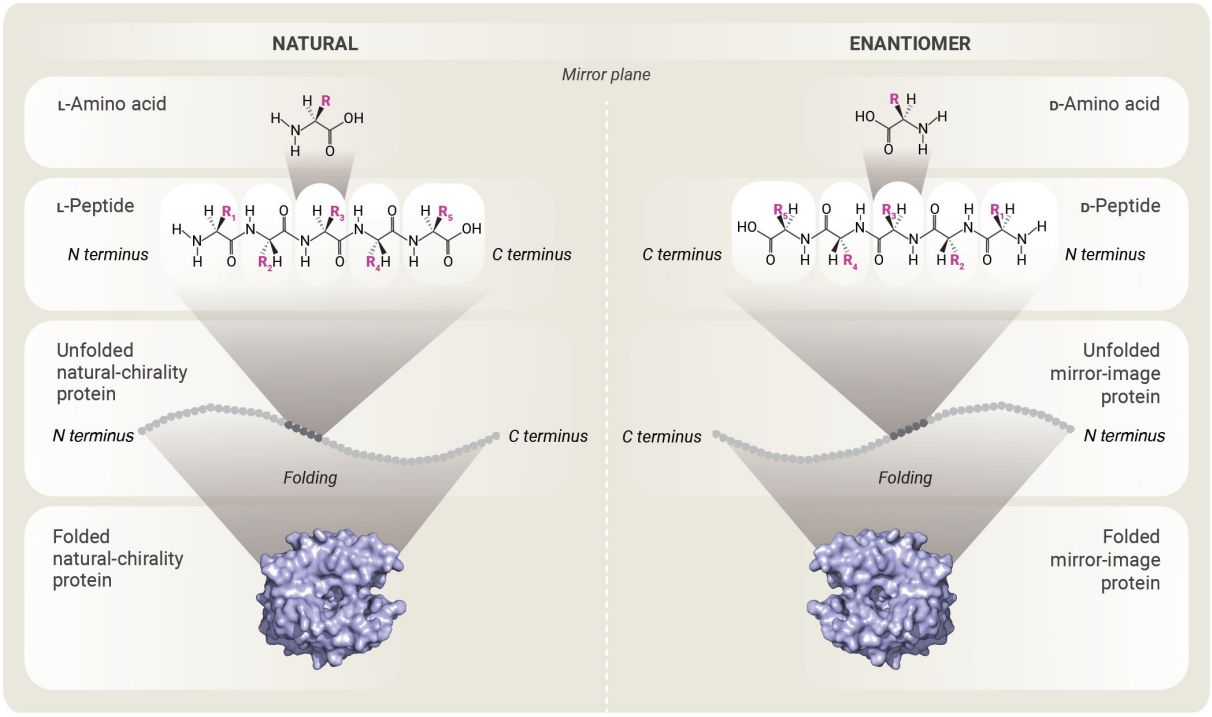

Figure 1.3: Mirror-image amino acids compose mirror-image peptides that fold into mirror-image proteins All natural amino acids except glycine are chiral, and only the L-enantiomers are used in natural proteins. Substituting the L-amino acids for their mirror image D-amino acid enantiomers would result in a mirror-image polypeptide chain. The interactions between the side chains for amino acids in the D-peptide are the same as the L-peptide, but with the opposite orientation. Thus, the mirror-image peptide chain folds into a protein that is the mirror image of its natural chirality counterpart. (Structure image for example protein, carbonic anhydrase, produced by Pymol 3.0.0 from Protein Data Bank accession number 1CA2.)

A mirror lifeform would be made entirely of mirror-image molecules, and as a consequence its interactions with natural biological organisms would be profoundly different. Just as a right-handed glove cannot fit a left hand, the molecules within a mirror bacterium would not "fit" the opposite-handed molecules found in nature. The natural-chirality receptors and enzymes of natural organisms would not properly bind to the mirror metabolites and macromolecules produced by a mirror bacterium, nor would the mirror-image receptors and enzymes in a mirror bacterium properly bind to natural-chirality metabolites and macromolecules.

Scientists, including several authors of this report, have long considered the possibility of creating mirror cells. One motivation comes from advances in synthetic chemistry, which have permitted the creation of progressively larger mirror nucleic acids and proteins. These mirror macromolecules retain the flexible programmability and functionality of natural biopolymers, but they resist degradation by nucleases and proteases and are much less likely to trigger undesired immune reactions—both properties that are desirable in therapeutic molecules. It is currently laborious to create mirror macromolecules synthetically, but increasingly accessible mirror nucleic acids and mirror enzymes will enable the creation of larger and more complex mirror molecular systems.

Independently, many synthetic biologists seek to understand how cells could be created from their constituent molecules. This is motivated both by a desire to better understand how life first arose, and

to understand what other kinds of life might be possible. If a natural-chirality cell can be created from lifeless molecules, then a mirror-image cell could be created from mirror-image molecules using the same methods.

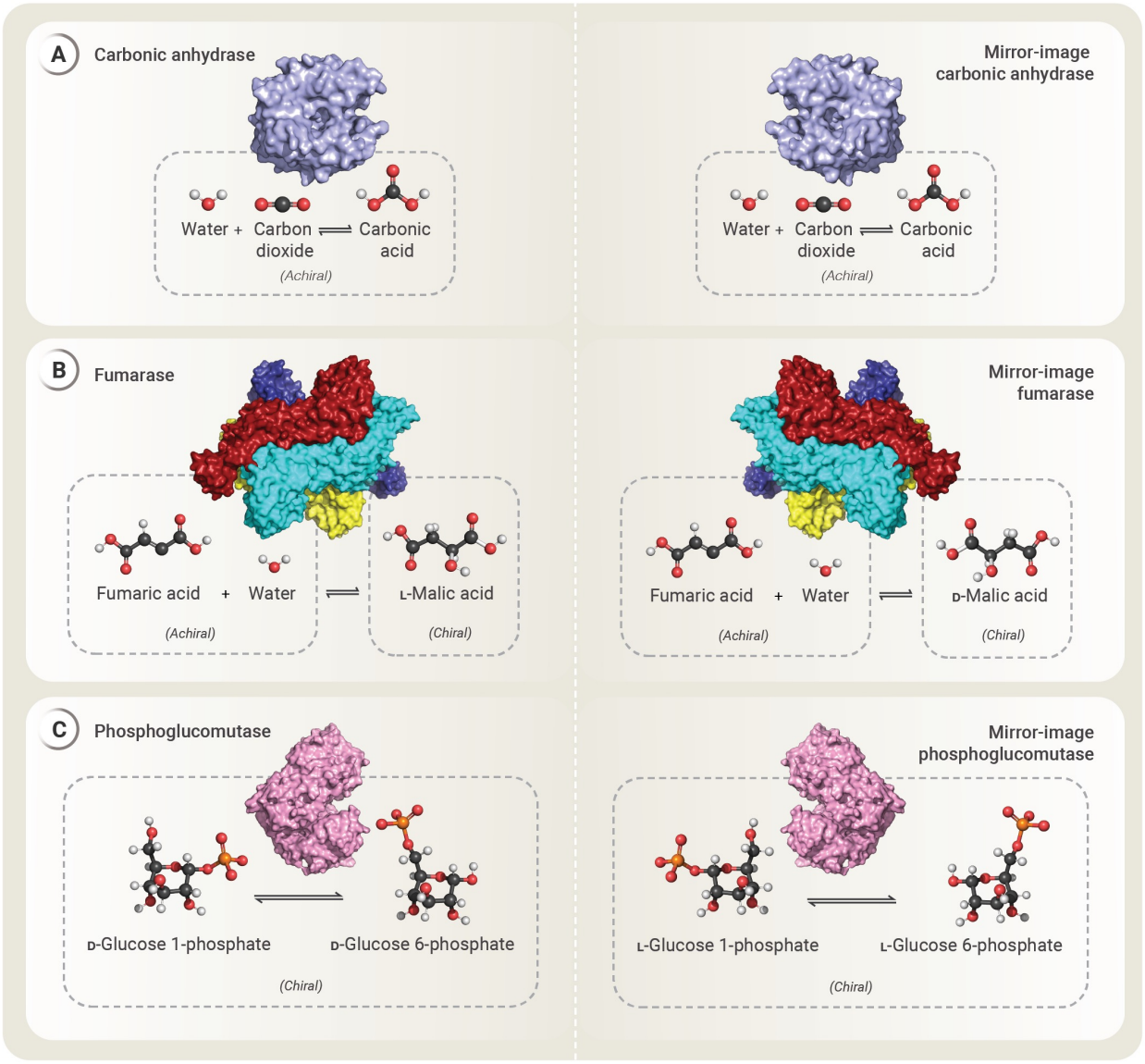

Figure 1.4: Mirror-image enzymes catalyze mirror-image reactions
Enzyme catalysis requires precise (and thus stereospecific) binding of substrate molecules to the active site. **A.** If all substrates and products of an enzyme are achiral, such as for carbonic anhydrase, the natural-chirality and mirror-image enzymes would behave identically. **B.** When the products of an enzymatic reaction are chiral, the natural-chirality enzyme exclusively produces one enantiomer and its mirror-image enzyme would produce the other. For example, both fumarase and mirror-image fumarase would produce malic acid from fumaric acid and water, but natural-chirality fumarase exclusively produces L-malic acid (the enantiomer seen in nature), while mirror-image fumarase would exclusively produce D-malic acid. **C.** When the substrate of an enzyme is chiral, catalysis is typically specific to that enantiomer. For example, natural-chirality phosphoglucomutase can convert D-glucose 1-phosphate to D-glucose 6-phosphate. In contrast, mirror-image phosphoglucomutase would convert L-glucose 1-phosphate to L-glucose 6-phosphate. (Structure images for carbonic anhydrase, fumarase, and phosphoglucomutase produced by Pymol 3.0.0 from Protein Data Bank accession numbers 1CA2, 3E04, and 3PMG respectively.)

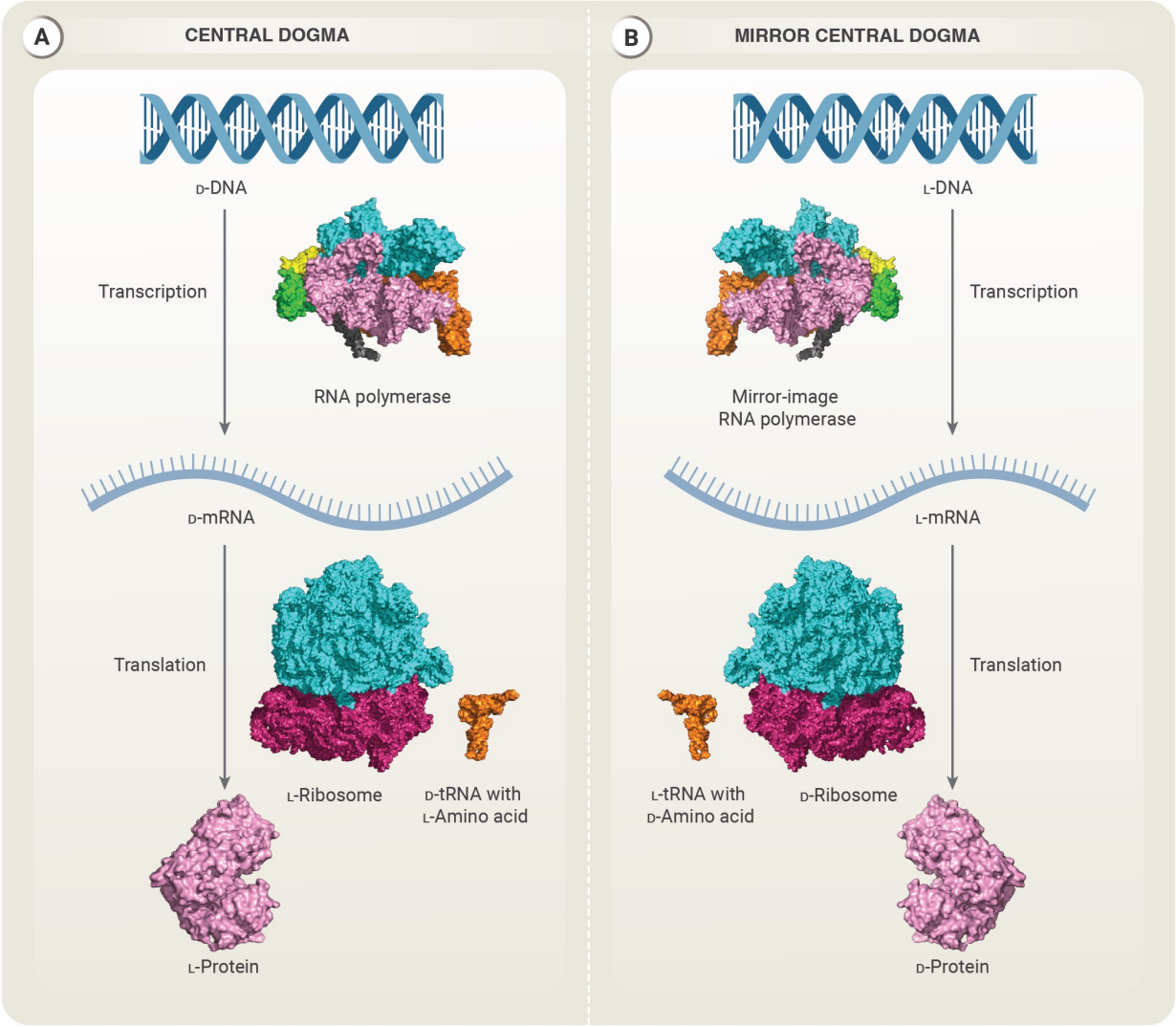

Figure 1.5: A mirror image of the central dogma
A. The central dogma of molecular biology. Genetic information in the form of D-DNA is transcribed by natural-chirality RNA to D-messenger RNA (D-mRNA), which is in turn translated into natural-chirality polypeptides and proteins. All of these stages exclusively use one of the two possible chiralities: natural chirality RNA polymerase transcribes D-DNA into D-mRNA, which the natural-chirality ribosome and transfer RNA (tRNA) carrying L-amino acids then translate into L-proteins. **B.** The central dogma could be chirally inverted: L-DNA would be transcribed by a mirror-image RNA polymerase into L-mRNA, which would be translated into mirror-image D-proteins. (Structure images for RNA polymerase, the ribosome, tRNA, and example protein (phosphoglucomutase) produced by Pymol 3.0.0 from Protein Data Bank accession numbers 4YG2, 5AFI, 6UGG, and 3PMG respectively.)

Once created, mirror cells could be further engineered using many of the same methods as natural-chirality organisms. Genes from natural-chirality organisms could be recreated with mirror-image DNA and engineered into mirror organisms, and the functionality of these genes would exactly mirror that of their natural counterparts. Diverse mirror lifeforms could thus be created by copying natural life. By perfectly mirroring existing life in isolation, but exhibiting greatly altered

interactions with natural organisms, mirror life could present an unprecedented and difficult to predict mixture of familiar and entirely novel behavior.

The authors of this report have come to the conclusion that mirror bacteria could present unique and severe risks. Their altered interactions with natural organisms could lead to profound and highly consequential effects on those natural organisms, as will be detailed in the subsequent chapters of this technical report.

The immune systems of humans, other animals, and plants primarily detect pathogens through the recognition of conserved chiral molecules. Recognition is stereospecific—it depends on the precise spatial arrangement of atoms in a molecule—and so the mirrored chiral molecules of mirror bacteria may not be properly recognized. Nor would antimicrobial enzymes or chiral antibiotics necessarily harm mirror bacteria, since they generally operate through chiral mechanisms. Protists and other predators could similarly have difficulty recognizing, killing, and digesting mirror bacteria. Mirror bacteria would also be entirely resistant to all natural bacteriophages. As a result, many if not most of the biological mechanisms that protect multicellular organisms from bacterial infection and limit bacterial populations in natural ecosystems would not function properly against mirror bacteria.

Like natural bacteria, mirror bacteria could be able to survive and replicate under diverse conditions. Some mirror bacteria may be fragile or lack the ability to synthesize required metabolites, just as is the case for many natural and engineered bacteria. But once the first mirror cells exist, creating more robust and diverse mirror bacteria should be straightforward and, absent concerns about potential risks, likely to be pursued by scientists seeking to advance the science and its practical applications. Such mirror bacteria could grow on achiral nutrients that are commonly found in many environments. They could also be engineered to consume D-glucose and other common chiral nutrients, and though there could be practical benefits to such engineering, this would also greatly facilitate growth in the environment.

If mirror bacteria could replicate within humans or other animals without being effectively cleared by the host immune system, they could establish a systemic infection with severe and plausibly fatal consequences. If that host or its carcass was consumed by another animal, the infection could spread, leading to transmission through the food chain. If mirror bacteria could replicate in soils and natural waters, but were not kept in check by natural enemies such as predators and phages, they would become invasive, severely disrupting natural ecosystems.

This chapter provides an overview of the entire report. Chapter 2 describes potential pathways scientists could pursue to create the first mirror bacterium. Chapter 3 then describes how mirror bacteria could be engineered to improve robustness and metabolic capabilities, which would raise serious biosafety and biosecurity challenges. Together these two chapters explain why, were it not for safety and security concerns, we think scientific progress over the next one to few decades could lead to the creation of mirror bacteria.

The remaining five chapters describe in detail the risks from mirror bacteria to humans, other animals, plants, and the environment more broadly. Chapter 4 describes the potential consequences of mirror bacteria for human health, detailing first the likely failures of both innate and adaptive immunity to recognize and respond to mirror bacteria before discussing the plausible course and

consequences of a systemic mirror bacterial infection. Chapter 5 then gives a brief overview of potential medical countermeasures that could protect humans from mirror bacteria, and the difficulties that developing and deploying such countermeasures would likely face.

Chapters 6 and 7 discuss other animals and plants, respectively. As with humans, serious immunological impairments could plausibly enable mirror bacteria to establish systemic infections within many animals and plants. Chapter 8 describes the survival and spread of mirror bacteria in the environment, both through and external to multicellular hosts, and closes by describing the irreversible ecological damage that an invasive mirror bacterium could cause.

Many aspects of our conclusions are necessarily tentative and uncertain. Natural organisms and ecosystems are complex and highly diverse, and any assessment of risk must extrapolate from limited information and a handful of examples subjected to detailed study. This single report, written at a single point in time with access to limited information on mirror biology, cannot be considered definite. We hope that others will build upon our initial analyses to examine these interdisciplinary risks in greater detail, potentially unearthing key considerations that we may have overlooked.

Mirror bacteria could plausibly be created in the next one to few decades

Many laboratories currently seek to achieve synthetic abiogenesis: the creation of life from its chemical building-blocks. The synthetic cell community is working to encapsulate self-replicating nucleic acids within a membrane and initiate metabolism under conditions that will trigger replication. Other biologists aim to simplify and then build synthetic versions of existing bacteria (Rothschild *et al*., 2024). As the smallest known bacterial genome size continues to decline—currently at half a million base pairs for engineered strains with reduced genomes (Hutchison *et al*., 2016)—the feasibility of creating a cell from scratch will grow.

Work toward mirror life is a natural outgrowth of work on synthetic cells. If scientists could construct a living bacterium from natural-chirality molecular precursors, the same methods could be used to construct a mirror bacterium from opposite-chirality precursors. While normal bioengineering often fails because the engineered alterations are incompatible with the rest of the organism, the processes for creating the first mirror cells could closely follow efforts to create natural-chirality cells. The first mirror cell could be an exact mirror-image of a pre-existing (albeit synthetic) natural-chirality cell.

Research on mirror-image proteins and nucleic acids is primarily motivated by potential near-term applications (see Boxes 2.1 and 2.2 in Chapter 2). Creating mirror life is a longer-term aspiration of multiple laboratories and major funders in an effort to better understand life and potentially aid in therapeutic development. For example, the U.S. National Science Foundation awarded $4 million to a group of investigators aiming “to design, construct, and safely deploy synthetic mirror cells in which all of the key molecules—nucleic acids, proteins, carbohydrates, and lipids—exist in chiral states opposite to their natural forms” (National Science Foundation, 2019a; National Science Foundation, 2019b). The National Natural Science Foundation of China has likewise supported work on the creation of a mirror-image central dogma (National Natural Science Foundation of China, 2022), and the European Commission’s ERA-NET MirrorBio consortium, with similar goals, was

Box 1.1: Violations of chiral symmetry are negligible in biological systems

Throughout this report we assume that mirror life would perfectly mirror natural-chirality life. If enantiomeric molecules perfectly mirrored one another, then it follows that the behavior of mirror bacteria would perfectly mirror that of natural-chirality bacteria. Conversely, if mirror bacteria did not perfectly mirror natural-chirality bacteria, this would imply the existence of some physical process that violates chiral symmetry between enantiomers.

Of the four fundamental forces in physics, only the weak force violates chiral symmetry. Any violation of chiral symmetry would result in a "parity-violation energy difference", or PVED, between enantiomers. Theoretical calculations for the weak force estimate a PVED of about 10^{-11} J/mol (Horný & Quack, 2015), which is about 10^{14} times smaller than thermal noise at room temperature, and 10^{16} times smaller than the strength of a typical chemical bond. It is therefore extremely difficult to see how such negligible effects could have any role in biology.

Attempts to experimentally observe differences between enantiomeric molecules have not yet succeeded (Quack *et al*., 2022; Sallembien *et al*., 2022). The most precise measurements to date, in CFHClBr, found no PVED at a sensitivity of $\sim 10^{-8}$ J/mol (Daussy *et al*., 1999). While experimental methods cannot yet detect the PVED from the weak force, these precision measurements, along with others in particle, atomic, and molecular physics (Chupp *et al*., 2019; Safronova *et al*., 2018) provide compelling evidence for the absence of other, as yet unknown, processes that could violate chiral symmetry at molecular scales.

Precision measurements focus on individual atoms and small molecules because they are the easiest to study; the possibility that parity violation would somehow become visible only in larger systems is remote. Studies of mirror-image proteins (Milton *et al*., 1992; Zawadzke & Berg, 1993) and mirror-image nucleic acids (Xu & Zhu, 2022) have not revealed any deviations from the expected mirror symmetry, nor have measurements of optical rotation angles or crystallographic structures of organic compounds provided any evidence of violations of chiral symmetry. It is therefore implausible that any hitherto unknown violation of chiral symmetry could play a role in biology, and so it is almost certain that a correctly constructed mirror life would essentially be a perfect mirror of natural life.

supported by the German Federal Ministry of Education and Research[3] and the U.S. National Science Foundation (National Science Foundation, 2014). We expect interest in mirror bacteria to grow as work on both synthetic cells and mirror-image macromolecules advances, which would make the task of building a mirror cell increasingly feasible.

[3] Bundesministerium für Bildung und Forschung grant 031A461

Estimating timelines is difficult. It is likely that normal-chirality synthetic cells are still years away, and that creating a synthetic mirror bacterium would take longer. Still, as detailed in Chapter 2, the foundational technologies are rapidly progressing, and there are multiple pathways by which the first mirror bacterium could be created.

The creation of any mirror bacterium could enable the creation of robust mirror bacteria

While creating the first mirror bacterium would be extremely challenging, the subsequent engineering of an existing mirror bacterium should be comparatively straightforward. Any bioengineering technique that can be accomplished by engineering a natural-chirality bacterium with normal DNA would apply to an equivalent mirror bacterium and mirror DNA. Even if the first mirror cells were fragile and catabolically limited—which is plausible but not certain—a logical next step would be to further engineer mirror bacteria in order to functionally mirror existing bacterial strains. A minimal and fragile mirror *Escherichia coli*, for instance, could be converted into the exact mirrors of widely used strains such as *E. coli* K-12, BL21, and Nissle 1917, and plausibly into more diverse bacterial species. Chapter 3 discusses the potential engineering of mirror bacteria in more detail.

Absent the severe risks discussed in this report, the mirror bacteria of greatest interest and utility would likely be mirrored versions of commonly studied heterotrophs such as *E. coli*, *Bacillus subtilis*, and *Pseudomonas aeruginosa*. These bacteria are useful, in part, because they are robust and can grow under diverse conditions. Because chirality would have little impact on robustness to abiotic stressors such as temperature, acidity, and oxidative stress, mirror versions of these heterotrophs would be similarly robust under diverse conditions. Given the likelihood that the first mirror bacteria would be a mirrored strain of *E. coli* or another species commonly used in the lab, a "generalist" *E. coli* is frequently used in this report as an illustrative example.

Mirror bacteria could acquire nutrients in natural environments

Sugars, amino acids, and other common nutrients are typically chiral. It might therefore appear that a mirror bacterium could not survive in the natural world: it would be unable to eat the available organic compounds and would thus starve. But this is not the case. Many common nutrients would be accessible to the mirrored versions of common heterotrophic bacteria, and with suitable engineering, additional nutrients like D-glucose could be made accessible.

As a concrete illustration, consider the example of an exact mirror *E. coli* K-12. The preferred carbon source for natural-chirality *E. coli* is D-glucose, so a mirror *E. coli* would prefer L-glucose, which is thought to be essentially absent from nature. However, natural *E. coli* can grow on a wide range of achiral substrates as sole carbon sources (see Table 1.1), including a number of central metabolites, fatty acids, and fermentation byproducts that are common in both multicellular hosts and in many external environments. A mirror *E. coli* should therefore grow as well as natural *E. coli* on these compounds.

There is nothing particularly exceptional about *E.* coli K-12 in this regard. The major metabolic pathways of life include a number of achiral metabolites, which are relatively abundant in nature and can be acquired and utilized relatively easily as nutrient sources. Some common achiral metabolites

Sole carbon sources		References
Central Metabolites	*Citrate,* fumarate, glycolate, glyoxylate, α-ketoglutarate, pyruvate, succinate	Clark & Cronan, 1996; Lin, 1996; Tong *et al*., 2020
Fatty Acids	Acetate, acetoacetate, *butyrate*, propionate, *valerate* *Medium-chain (C_6–C_{10}) fatty acids* Long-chain ($\geq C_{12}$) fatty acids	Clark & Cronan, 1996; Clark & Rod, 1987
Alcohols	*Butanol*, *ethanol*, *propanol*	Clark & Cronan, 1996; Clark & Rod, 1987
Sugars and polyols	Dihydroxyacetone, *ethylene glycol*, galactitol, glycerol, mucate	Lin, 1996; Tong *et al*., 2020
Aromatic Acids	*Benzoate*, m-coumarate, *2-furoate*, *3-hydroxyphenylacetate*, phenylacetate, phenylpropionate, phenylethylamine	Abdulrashid & Clark, 1987; Díaz *et al*., 2001
Amines	*γ-aminobutyrate*, *putrescine*	Dover & Halpern, 1972; Prieto *et al*., 1987
Miscellaneous	γ-hydroxybutyric acid, methyl pyruvate, m-tartaric acid	Tong *et al*., 2020
Sole nitrogen sources		
Amino Acids	Glycine	Reitzer, 2005
Amines	Agmatine, γ-aminobutyrate, dopamine, phenylethylamine, putrescine, spermidine, tyramine	Díaz *et al*., 2001; Reitzer, 2005
Nucleobases	Adenine, cytidine, thymine, uracil Allantoin*, urate*	Cusa *et al*., 1999; Iwadate & Kato, 2019; Tyler, 1978; Xi *et al*., 2000

Table 1.1: Achiral organic molecules that can be utilized by wild-type or mutant *E. coli* K-12
E. coli K-12 can use a number of achiral organic compounds as either sole carbon or nitrogen sources. Entries in *italics* indicate use by mutants. *Can only be utilized as a nitrogen source under anaerobic conditions.

cannot be utilized as a sole carbon source by natural *E. coli* K-12, but can be used by other bacteria. These include ethanol, glycine, inositol, methanol, and ribitol, although in all five cases strains of *E. coli* K-12 have been engineered to grow on these compounds as sole carbon sources (Cao *et al*., 2020; Fung *et al*., 2023; Keller *et al*., 2022; Scangos & Reiner, 1978; Shiue & Prather, 2014).

A number of chiral nutrients could also be utilized by mirror *E. coli* K-12 to varying extents. Table 1.2 compares the catabolic utilization of L- and D-amino acids by natural-chirality *E. coli* K-12. Although amino acids of the wrong chirality cannot be incorporated directly into proteins by the ribosome, they can be degraded to provide energy and useful nutrients. A mirror *E. coli* could utilize seven of the canonical amino acids to some extent, and the ability to utilize a further eight could quickly arise through mutations (see Section 8.4 for further discussion of mirror bacterial evolution). Most notably, natural-chirality *E. coli* catabolizes L-alanine through D-alanine and can use either as a sole carbon and nitrogen source (Reitzer, 2005). A mirror *E. coli* would therefore have no difficulty catabolizing L-alanine, which is among the most abundant amino acids. Other common chiral

nutrients that could be utilized by mirror *E. coli* include lactic, malic, and tartaric acid, as natural *E. coli* can catabolize both enantiomers of these chiral compounds (Dong *et al*., 1993; Lukas *et al*., 2010; Unden *et al*., 2016).

An exactly mirrored *E. coli* could not catabolize D-glucose, but engineering mirror *E. coli* to catabolize D-glucose should be feasible. Genes for L-glucose catabolism have been identified from α-proteobacteria *Paracoccus laeviglucosivorans* (Shimizu *et al*., 2012); while this ability has not yet been engineered into a natural-chirality *E. coli*, there appears to be no inherent barrier to doing so. Plausible catabolic pathways for other monosaccharides also exist (see Table 1.3). Mirror *E. coli* would also exhibit chemotaxis toward common sugars, though less effectively than natural-chirality *E. coli* (Adler *et al*., 1973).

Amino Acid	**L-isomer**	**D-isomer**	**References**
Alanine	Sole C and N source	Sole C and N source	
Arginine	C source, sole N source	*Utilize*	Celis, 1977
Asparagine	Sole C and N source		
Aspartate	Sole C and N source		
Cysteine	C source, sole N and S source	C and N source, sole S source	Soutourina *et al*., 2001
Glutamine	C source, sole N source		
Glutamate	*C source*, sole N source	Utilize in cell wall	Dougherty *et al*., 1993
Histidine	Utilize	*Utilize*	Kuhn & Somerville, 1971
Isoleucine	Utilize	*Utilize*	Kuhn & Somerville, 1971
Leucine	Utilize	*Utilize*	Kuhn & Somerville, 1971
Lysine	Utilize		
Methionine	Utilize	*Utilize*	Kadner, 1977
Phenylalanine	Sole C and N source	*Utilize*	Kuhn & Somerville, 1971
Proline	Sole C and N source		
Serine	Sole C and N source	C source, sole N source	
Threonine	C source, sole N source	Utilize to synthesize isoleucine	Umbarger & Brown, 1956
Tryptophan	C source, sole N source	*Utilize*	Kuhn & Somerville, 1971
Tyrosine	Utilize	*Utilize*	Kuhn & Somerville, 1971
Valine	Utilize	*Utilize*	Kuhn & Somerville, 1971

Table 1.2: Utilization of L- and D-amino acids by *E. coli*
Entries in *italics* indicate use by mutants. Citation to (Kuhn & Somerville, 1971; Reitzer, 2005) unless otherwise indicated. For some D-amino acids we were unable to find studies of their utilization by *E. coli*, though D-asparagine, D-aspartate, and D-lysine cannot be used as a sole nitrogen source (Bochner *et al*., 2001). Glycine, which is achiral, is absent from the table; it can be used as a carbon source and as a sole nitrogen source.

Monosaccharide	Utilization by *E. coli*	Potential pathways	References
Hexoses			
L-fructose	No	Through isomerase to L-glucose	Park *et al*., 2007
L-galactose	By mutation	L-fucose regulatory mutation and selection	Zhu & Lin, 1986
L-glucose	No	Through L-gluconate to pyruvate and GAP	Shimizu *et al*., 2012
L-mannose	No	Through isomerase to L-fructose	Park *et al*., 2007
Pentoses			
D-arabinose	By mutation	L-fucose regulatory mutation and selection	LeBlanc & Mortlock, 1971
L-ribose	By mutation	Through mutation and selection	Trimbur & Mortlock, 1991
L-xylose	No	Through isomerase to L-xylulose	Usvalampi *et al*., 2012
L-xylulose	Yes	Native ability	Ibañez *et al*., 2000
Trioses			
L-glyceraldehyde	By mutation	Through glycerol-3-phosphate Through glycerol	Baldomà & Aguilar, 1987; Kalyananda *et al*., 1987; Zhu & Lin, 1987

Table 1.3: Utilization of the enantiomers of common monosaccharides by *E. coli*
E. coli is able to utilize the enantiomers of a number of common sugars. Note that unlike most sugars, L-arabinose is the form most commonly found in nature.

Catabolizing larger sugars such as L-sucrose or L-trehalose might be more challenging, as it is unclear whether suitable transporters or hydrolytic enzymes exist in nature. Nevertheless, there is no underlying principle that would prohibit engineering a mirror bacterium with rich and varied catabolic capabilities; absent concerns about the risks, such work could proceed rapidly.

In addition to organic carbon, heterotrophic bacteria also require inorganic nutrients, but these are almost always available in achiral forms. Like most bacteria, *E. coli* can acquire nitrogen from inorganic ammonium, nitrate, and nitrite, and from achiral organic compounds such as glycine and urea. Phosphorus and sulfur are likewise available as phosphate and sulfate, while chlorine and metals are available as monatomic ions. Indeed, an organic carbon source like D-glucose is often the only chiral nutrient used in minimal culture media.

It appears that many environments could have suitable abiotic conditions and sufficient nutrients to support growth of a mirror *E. coli* or other similarly robust mirror bacterium; we discuss specific environments in more detail in Chapters 4, 6, 7, and 8. It is conceivable that some common chiral compounds could be toxic to mirror bacteria, but as discussed in Box 1.2, this does not appear to be a significant impediment. Thus, there do not appear to be any fundamental barriers preventing the survival and replication of mirror bacteria in many natural environments.

Box 1.2: Could natural-chirality molecules be toxic to mirror life?

The organic compounds found in natural environments are generally not toxic to bacteria. But for a mirror bacteria containing mirror enzymes and other mirror macromolecules, it is conceivable that toxicities could arise. This possibility can be addressed by considering the equivalent "mirrored" question: are the enantiomers of common organic compounds toxic to natural bacteria?

D-amino acids are the most widely studied class of mirror metabolites. Studies have found that some can inhibit bacterial growth, but they typically require 1–10 mM concentrations for even mildly inhibitory effects (Kao *et al*., 2017; Rumbo *et al*., 2016; Sarkar & Pires, 2015; Soutourina *et al*., 1999; Soutourina *et al*., 2000). L-amino acid concentrations in most natural environments are typically much lower, and therefore would be unlikely to inhibit mirror bacterial growth.

A few D-amino acids are particularly toxic. D-tryptophan has been reported to mildly suppress *E. coli* growth at low concentrations (Soutourina *et al*., 2000). However, given that *E. coli*, *Acinetobacter baumannii*, and *P. aeruginosa* can all sustain growth even at 5 mM (Rumbo *et al.*, 2016; Soutourina *et al*., 2000), naturally occurring concentrations of L-tryptophan are unlikely to significantly inhibit mirror bacterial growth. Toxicity is likely caused by the accidental incorporation of D-tryptophan into proteins. D-amino acid tRNA deacylases, which are present in nearly all living organisms (Wydau *et al*., 2009), protect against misincorporation toxicity by eliminating mischarged tRNAs. Simply upregulating this enzyme would likely provide mirror bacteria with additional protection for a mirror bacterium.

D-cysteine can likewise mildly suppress *E. coli* growth at micromolar concentrations in minimal media, although it has no inhibitory effects at even 10 mM in LB broth (Soutourina *et al*., 2001). D-cysteine and D-cystine are actively transported into the cytosol through transporters for L-cysteine and L-cystine (Chonoles Imlay *et al*., 2015; Zhou & Imlay, 2022). Toxicity appears to be primarily driven by inhibition of threonine deaminase, needed for the biosynthesis of isoleucine, leucine, and valine, as addition of these amino acids to minimal media reduces (though does not entirely alleviate) growth inhibition by D-cysteine (Soutourina *et al*., 2001); L-cysteine is toxic through a similar mechanism (Sørensen & Pedersen, 1991). D-cysteine is detoxified by D-cysteine desulfhydrase, which decomposes it into pyruvate, ammonium, and hydrogen sulfide; upregulation of this enzyme increases the resistance of *E. coli* to D-cysteine toxicity (Soutourina *et al*., 2001).

The extent to which L-cysteine would pose a barrier to growth of an unadapted mirror *E. coli* within living organisms is unclear. D-isoleucine, D-leucine, and D-valine would not be present in natural environments, so mirror-image *E coli* would likely be reliant on its mirror-image threonine deaminase for biosynthesis, and so plausibly could be susceptible to

growth inhibition by L-cysteine. Resistance to L-cysteine could rapidly emerge, for instance through mutations to the transporters or by upregulation of D-cysteine desulfhydrase. Utilization of naturally occurring amino acids could also obviate the need for threonine deaminase (see Table 1.3), and *E. coli* strains lacking threonine deaminase may still produce isoleucine through other metabolic pathways (Cotton *et al*., 2020). It is plausible that L-cysteine would have similar inhibitory effects on other mirror bacterial strains, though not universally; *P. aeruginosa*, for instance, is uninhibited at millimolar concentrations of D-cysteine (Rumbo *et al*., 2016).

To our knowledge, the enantiomers of common sugars are generally not toxic to bacteria. For *E. coli* the only exception we have found is L-glyceraldehyde, which can mildly inhibit *E. coli* growth at millimolar concentrations (Tang *et al*., 1977). It appears, however, that toxicity is due to the oxidation of L-glyceraldehyde to L-glycerate, as *E. coli* mutants lacking aldehyde dehydrogenase can grow on L-glyceraldehyde (Zhu & Lin, 1987). The mechanism by which L-glycerate is toxic to *E. coli* is not clear, but glyceric acid is not particularly abundant in nature.

We have not found other plausible chiral molecules that would preclude mirror *E. coli* growth in natural environments. Not all enantiomers of common molecules have been tested and reported in the literature, and it is possible we have missed other known toxicities. Nevertheless, it appears unlikely that any natural-chirality organic compounds would prove unusually toxic to most mirror bacteria at the very low concentrations typically present in most environments.

Mirror bacteria could be innately resistant to many immunological mechanisms

Biochemically, the interiors of multicellular organisms are generally nutrient-rich and growth-permissive for many natural bacteria. Sufficiently robust mirror bacteria would similarly be capable of replicating within multicellular organisms by utilizing achiral nutrients and, with suitable engineering, common sugars such as D-glucose. Plants and animals have evolved sophisticated immune systems to protect themselves from bacterial infection. But both the innate immune receptors and the immune effectors that cause harm to bacteria are generally chiral, having evolved to target conserved chiral molecules specific to natural-chirality bacteria. Therefore, it is likely that many immunological mechanisms would not function effectively against mirror bacteria because they could not recognize the enantiomers of their targets.

Plant and animal immune systems, for example, possess pattern-recognition receptors (PRRs) that detect the presence of microbe-associated molecular patterns (MAMPs). While the specific PRRs vary between species, they typically recognize highly conserved MAMPs, such as lipopolysaccharides and peptidoglycans that are present in many or all bacteria. Almost all such MAMPs are chiral. Because interactions between PRRs and MAMPs are stereospecific, PRRs would not typically recognize mirror lipopolysaccharides, mirror peptidoglycan, or the other

opposite-chirality macromolecules produced by mirror cells. There could be exceptions: at least one achiral MAMP is known to be recognized by a human PRR (Uldrich *et al*., 2020) and another in plants (Kakkar *et al*., 2015), though both appear to have only marginal importance. Some PRRs might primarily recognize achiral regions of their targets and retain weak affinity for the enantiomer, and unexpected cross-reactivities could also trigger PPRs. Moreover, innate immune systems detect damage-associated molecular patterns (DAMPs) caused by cell death, but these are unlikely to be triggered until late in an infection and appear unlikely to help the immune system target and eliminate infecting mirror bacteria. Failures of innate immune recognition could seriously impair immunological responses to mirror bacterial infection, as discussed in Chapters 4, 6, and 7.

Humans and other vertebrates also possess an adaptive immune system, which is theoretically capable of generating antibodies against any novel pathogen and could in principle produce antibodies that would bind to mirror-image macromolecules. But as discussed in Section 4.3, many of the steps required to process and present antigens for T cell activation are stereospecific and would likely fail, severely limiting B cell activation and the production of antibodies. An MHC-II immune deficiency is perhaps the closest clinical analog, and in such patients the antigen-specific T and B cell response is profoundly impaired.

Impairments in immunological recognition would be further compounded by the inability of some, though not all, immune effectors to harm bacteria. Antimicrobial enzymes such as lysozyme, phospholipases, and proteases target peptidoglycan, phospholipids, and proteins, all of which are chiral; these enzymes are unlikely to retain significant activity against their mirror counterparts. Other antimicrobial peptides cause harm by disrupting membranes and, in at least some cases, would retain efficacy against mirror bacteria. Within vertebrates, the alternative complement pathway may still function, but other complement activation pathways could be more significantly impaired. The extent to which professional phagocytes could successfully internalize and kill mirror bacteria is unclear.

Chapters 4, 6, and 7 discuss these immunological failures, and the consequent possibility of mirror bacterial infection in humans, animals, and plants in more detail. In all cases, physical barriers are imperfect and allow for at least occasional bacterial translocation into vulnerable parts of the organism. Given the extent to which mirror bacteria would innately evade many immunological mechanisms, it appears plausible that a sufficiently robust mirror bacterium would be capable of entering and harming many multicellular hosts, including humans.

While mirror bacteria would lack the toxins or other virulence factors typically produced by natural pathogens, uncontrolled mirror bacterial growth within a multicellular host could prove dangerous. In humans, for instance, a systemic mirror bacterial infection might prove fatal through a sepsis-like severe immunological dysregulation (see Section 4.5). Even if this were avoided, a bloodstream mirror bacterial infection could likely deplete nutrients, modify blood chemistry, and cause physical disruptions to blood vessels and organs, all of which could be lethal.

Mirror bacteria could be innately resistant to most forms of predation

Chapter 8 discusses potential ecological risks from mirror bacteria. In the natural environment, bacterial populations are often kept in check by protists, phages, and other predators. Mirror bacteria would be completely resistant to natural phages, which could neither adsorb to mirror receptors nor replicate their genomes with mirror polymerase. Mirror bacteria may also avoid most predation from protists, which recognize bacterial prey through receptors that rely on stereospecific interactions. While the mechanisms of prey recognition are not well understood, plausible failures of protist receptors to recognize mirror metabolites and macromolecules would impair both tracking and engulfment of mirror bacteria. Even if ingested, it is not clear how effectively protists would kill mirror bacteria. It is clear, however, that the mirror macromolecules would not be digested by the natural-chirality hydrolases produced by protists. Protists would gain few, if any, nutrients from mirror bacteria and thus would be unlikely to evolve to consume them more effectively. Other bacterial predators such as nematodes and filter feeders could likely face similar challenges.

Although a robust mirror bacterium could potentially replicate in many environments, any given mirror strain would be unlikely to replicate as rapidly as natural bacteria in the same environment because it would not be able to utilize the full range of chiral nutrients available to natural organisms. Moreover, any single mirror strain would initially be poorly adapted to most of the environments it might encounter.

However, for a mirror strain to become established within a natural environment merely requires the mirror bacteria to replicate faster than they die. Replication rates of mirror bacteria relative to natural bacteria could be lower, but so could mortality rates, as mirror bacteria are likely to evade many forms of predation. Slowly growing mirror bacteria might therefore be able to invade some ecosystems.

An expanding mirror bacterial population would face no commensurate expansion in phages or predation because phages would not infect mirror bacteria and predators would gain few, if any, nutrients from consuming them. As a result, mirror bacterial populations could become large in successfully colonized environments, with growth ceasing only once the available nutrients became sufficiently depleted.

Invasive mirror bacteria could spread and rapidly adapt to new environments, causing severe ecological harms

A mirror bacterium capable of infecting multicellular organisms could spread through varied means: predation of infected animals and scavenging of infected corpses, insect bites and similar vectors, consumption of infected plants, and exposure to contaminated soil or water. It is likely that a mirror bacterium capable of infecting many multicellular species would undergo sustained transmission through the environment.

Natural pathogens can spread many kilometers each day if transported by insects, birds, or other rapidly dispersing hosts. If mirror bacteria were able to infect such animals, they would likewise

Box 1.3: Why is mirror life absent from nature?

The risks from mirror bacteria arise because reversed-chirality confers significant advantages through immune evasion and predation resistance. If reversed-chirality could be so advantageous, why does mirror life appear to be absent from nature?

Evolution is a stepwise process involving the gradual accumulation of advantageous mutations. If it was possible to invert the chirality of one surface molecule at a time, each of which would confer some resistance, then we might see such changes in natural organisms. But inverted chirality appears to be all-or-nothing: either a cell can make mirror proteins, and therefore other mirror macromolecules, or it cannot. There is simply no plausible series of stepwise changes through which a natural-chirality cell could build functional mirror proteins without severely damaging its ability to make normal proteins. Even small alterations to the genetic code alter the structure of all existing proteins, likely causing numerous fatal changes (Crick, 1968). As a result, the genetic code has remained remarkably conserved across living organisms (Ambrogelly *et al*., 2007; Koonin & Novozhilov, 2017). Modifying an existing cell into a mirror cell would require many steps of similar or greater complexity to the incorporation of a novel amino acid, and therefore appears to be essentially impossible.

In principle, mirror life could arise independently of existing life, but there is also no compelling reason to think that mirror life would have arisen independently of ordinary life. Opinion varies as to whether the origin of life was an extremely rare event, almost inevitable, or something in between; the very limited evidence available does not allow strong conclusions to be drawn on this matter (Spiegel & Turner, 2012). If abiogenesis is rare, it would have been very unlikely for life to have arisen twice, and so mirror life probably never arose on Earth. Even if abiogenesis happened more than once on the early Earth, it is not clear that other living organisms would have had a close resemblance to modern life, mirrored or otherwise.

It thus appears most likely that nothing similar to a mirror bacterium ever existed on Earth. The absence of mirror life from nature provides no cause for reassurance with respect to synthetic mirror life.

spread at a similar speed (see Section 8.3). Human activity could spread invasive mirror bacteria even faster via airplanes, cargo ships, and road vehicles.

Mirror bacteria that escape containment would likely start with essentially no standing genetic variation. They would have no ability to acquire genes by horizontal transfer from existing bacteria, because the mirrored molecular machinery of a mirror bacterium could not read natural-chirality DNA. As they spread, they would encounter diverse environments they are poorly suited for, but mutation and natural selection would allow them to adapt (see Section 8.4). Invasive mirror bacteria could thus diversify into multiple lineages, specializing and evolving to colonize previously

inaccessible environments and infect novel hosts. Even if an escaped mirror bacterium could initially survive only under restricted conditions, it could subsequently evolve increased fitness and an expanded range.

An invasive mirror bacterium able to infect many multicellular hosts could plausibly drive most susceptible animal and plant species to extinction owing to repeated infections from abundant mirror bacterial populations in the external environment. The loss of many species simultaneously would be highly disruptive to natural ecosystems, particularly if ecologically critical species are susceptible. A mirror photoautotroph would be particularly disruptive to marine ecosystems because it would not require any chiral nutrients to thrive.

Creating biocontained mirror bacteria may be feasible, but securing them against misuse appears exceptionally challenging

If mirror bacteria are created, it would be relatively straightforward to engineer more robust and diverse mirror strains and species, which could pose serious risks to both human health and the environment. The creation of any mirror bacterium would thus create serious biosafety and biosecurity challenges. The technical feasibility of containment strategies to mitigate risks are discussed in Chapter 3.

Creating a "biocontained" mirror bacterium—that is, one incapable of replicating outside of a laboratory—should be possible. For instance, a metabolic auxotroph that lacked the biosynthetic pathways for pantothenate and biotin would be able to grow only if provided mirror pantothenate and mirror biotin, both of which are likely absent from nature. Given the potentially severe consequences of an escape, any biocontainment methods used with mirror bacteria would have to be extremely robust to all possible paths to escape, including failures arising from human error.

Most concerningly, even if robust biocontainment were possible, such measures could always be undone by malicious or reckless actors. Given the relative ease with which mirror bacteria could be engineered if created, engineering versions that could not be abused by malicious actors appears to be extraordinarily difficult.

Countermeasures could mitigate some harms, but could not prevent irreversible environmental damage

Chapter 5 describes potential medical countermeasures that could be used to protect humans from mirror bacteria. While most existing antibiotics are chiral and interact stereospecifically with their microbial targets, those antibiotics that are achiral would still prove harmful to mirror bacteria; novel "antimirror" compounds could also be identified and produced. The clinical efficacy of such antimirror compounds would be unclear until tested, and scaling production to sufficiently protect all humans would be a challenging, and perhaps impossible, undertaking. Other therapeutics such as conjugate vaccines, antibody therapy, and phage therapy appear to be technically feasible, but the practical challenges to developing such measures quickly and at scale would be considerable.

Protecting agricultural production against invasive mirror bacteria could require genetically engineering resistant crops or cultivating resistant microbes or simple plants as alternative food sources (see Section 7.4). Protecting most wild plants or animals through similar engineering would not be feasible. Biological countermeasures such as mirror phages, discussed in Section 8.6, could in principle reduce mirror bacterial populations after they become established. Like existing biocontrol measures, however, they would be unable to completely eliminate mirror bacteria from ecosystems, and so could not protect plants and animals from repeated exposure and infection. If an invasive mirror bacterium were inadvertently or maliciously released into the environment, there does not appear to be any realistic prospect for averting irreversible and widespread environmental harm.

Chapter 2: Pathways to Mirror Life

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Ever since Louis Pasteur recognized the chiral nature of life, scientists have speculated about the possibility of mirror-image life forms. Mirror-image organisms would be expected to share the properties of their natural-chirality counterparts, akin to the similarities between left and right hands. However, they could not emerge by natural evolution from existing natural-chirality life: this would require the simultaneous inversion of the chirality of all mirror biomolecules, which is incompatible with the gradual nature of evolution (see Box 1.3). The construction of entirely new forms of life in the laboratory has long seemed like a distant prospect, but advances in synthetic chemistry and synthetic biology make the human-led creation of mirror-image organisms increasingly realistic in the coming decades.

In this chapter, we discuss key enabling technologies and potential paths through which a mirror bacterium might be created based on our current understanding. We do not expect that the creation of a mirror bacterium, if further pursued, would follow exactly one of the outlined strategies, but the analysis here may be illustrative to understand the overall feasibility, as well as the challenges and strategies that would be involved in a future effort. As we will show, plausible paths to the creation of a mirror bacterium exist, but numerous technical hurdles remain to be overcome. Overall, the creation of a mirror bacterium will require extensive research. We estimate that if substantial resources were invested in a concerted effort, the creation of a mirror bacterium might still be 10 years away; and if research continues on its current trajectory, mirror bacteria might be created in the next 15 to 30 years.

Mirror bacteria could present novel and severe biosafety and biosecurity challenges, as outlined in the other chapters of this report. By default, many of the bottlenecks toward the creation of mirror cells will likely be addressed by scientists attempting to solve related problems, even if these researchers have no explicit interest in creating mirror bacteria. This chapter aims to assess the feasibility of the creation of mirror bacteria in the wider context of ongoing scientific advances and highlight the technical hurdles, which might serve as an indicator of progress toward mirror life. Discussion of governance options is out of the scope of this report.

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Section 2.1 provides an overview of recent advances in the chemical synthesis of mirror biomolecules. Chemical synthesis, unlike biological synthesis, is equally well suited to create natural-chirality or mirror-image biomolecules such as nucleic acids and proteins. Advances in chemical synthesis have recently enabled the synthesis of mirror-image DNA and RNA at the kilobase-scale, and the synthesis of large mirror-image proteins up to 100 kDa; in principle, this is sufficient to create most components of a mirror bacterium. Ongoing research on improving these methods is expected to lower the cost and complexity further, making mirror biomolecule synthesis more accessible.

Section 2.2 considers how, leveraging the advances in chemical synthesis, a mirror bacterium could be created "bottom-up". While it is not yet possible to create living cells from non-living components of any chirality, bottom-up synthetic cell research is rapidly advancing, and a subset of the methods could be adapted to create mirror cells in the future. A plausible strategy to create a mirror bacterium bottom-up would involve creating a mirror genome and mirror transcription and translation machinery by chemical synthesis and then encapsulating them in a synthetic mirror membrane. Under the right conditions, the mirror transcription and translation machinery might then be used to express a mirror transcriptome and proteome from the mirror genome, to "boot up" a self-replicating mirror cell.

Section 2.3 considers an alternative path to the creation of a mirror cell, the stepwise conversion of a natural-chirality bacterium into a mirror bacterium. Extensive work in the field of genetic code expansion and reprogramming is enabling researchers to make proteins *in vivo* with non-canonical amino acids beyond the canonical twenty. Building on this work, it might be possible to create a "crossover" translation pathway in which natural-chirality bacteria are able to produce mirror proteins using natural-chirality machinery. The crossover translation system could be used to create a self-sustaining mirror central dogma within the cell. Finally, a synthetic mirror genome could be delivered to this mixed-chirality cell and the natural-chirality genome removed, to create a fully mirrored bacterium. While this pathway might appear more challenging than the approach discussed in Section 2.2, it might be differentially accelerated by advances in synthetic biology and translational engineering over the coming decades.

Section 2.4 discusses synergies between these two conceptual approaches and additionally considers more speculative pathways. Scientific progress is hard to predict, and as different areas of science advance, alternative pathways might become increasingly plausible. For example, the development of abiological artificial cells could provide the basis for mirror cell research in the future. Advances in computational or experimental methods that would greatly improve our ability to create designer enzymes could enable alternative pathways to mirror life.

Section 2.5 synthesizes the major bottlenecks along each pathway and discusses the overall feasibility of creating mirror bacteria.

2.1 Advances in chemistry permit the synthesis of mirror biomolecules with diverse applications

Historically, most biological macromolecules such as proteins or nucleic acids were derived from living organisms. As a result, only their natural-chirality versions have been accessible for applications in basic science and biotechnology. Advances in chemical synthesis permit the creation of proteins and nucleic acids of either chirality, enabling new applications of these mirror biomolecules.

Progress in chemical peptide and protein synthesis is making mirror-image peptides and proteins more accessible

The creation of a mirror bacterium would require the synthesis of a large number of mirror-image proteins. Peptides of both chiralities are routinely made by solid-phase peptide synthesis (SPPS) (Behrendt *et al*., 2016). In this process, the starting amino acid is covalently attached to a solid polymer support through its carboxyl group and additional amino acids are added in a stepwise fashion. This works equally well for natural-chirality and mirror-image polypeptides and typically permits the synthesis of polypeptides of 30–60 amino acids, although longer polypeptides can sometimes be made under optimized conditions. Building on this method, the first synthesis of a mirror-image protein was achieved by Kent and colleagues, who in 1992 created a 99-amino-acid mirror-image HIV-1 protease entirely by SPPS and demonstrated that—as expected—the mirror-image protease was capable of cleaving a mirror-image peptide substrate but not the natural-chirality substrate (Milton *et al*., 1992). Modern SPPS can be automated, and improving methods enable the synthesis of increasingly challenging and long polypeptides. For example, the recent development of fast-flow automated peptide synthesis devices has permitted the production of small proteins of up to 164 amino acids, including several small mirror-image proteins, within a few days (Callahan *et al*., 2024; Hartrampf *et al*., 2020).

The development of native chemical ligation has substantially increased the size and complexity of proteins that can be made by total chemical synthesis (Kulkarni *et al*., 2018). This approach involves synthesizing several peptide fragments of 30–60 amino acid length by SPPS and then ligating these peptides together to form the full-length protein (X. Lin *et al*., 2023). Successes include the total synthesis of the mirror image of the 312-amino acid 4-hydroxy-tetrahydrodipicolinate synthase from *Escherichia coli* (DapA) (Weinstock *et al*., 2014) and the 352-amino acid DNA polymerase IV from *Sulfolobus solfataricus* (Dpo4) (Pech *et al*., 2017; W. Xu *et al*., 2017).

Recently, the chemical synthesis of the mirror image of the 775-amino acid DNA polymerase from *Pyrococcus furiosus* (*Pfu* DNA Pol) (Fan *et al*., 2021), and the 883-amino acid T7 RNA polymerase (T7 RNAP) (Y. Xu & Zhu, 2022) have also been accomplished. Since these proteins are too large to be made even with native chemical ligation, the authors performed rational engineering of the enzymes to generate split proteins. The synthesis of *Pfu* DNA Pol was achieved by splitting the enzyme into a 467-amino acid and a 308-amino acid subunit. T7 RNAP was made by splitting the enzyme into a 363-amino acid, a 238-amino acid, and a 282-amino acid subunit. The authors additionally introduced point mutations to remove hydrophobic residues to increase the solubility of

Box 2.1: Applications of mirror peptides and proteins

Peptides are promising **therapeutics** due to their ability to bind drug targets with high affinity. However, natural-chirality peptides suffer from comparatively low biostability as they can be degraded by proteases (enzymes that degrade peptides and proteins) that are abundant in most living organisms and play important roles in the immune system (Muttenthaler *et al*., 2021). Scientists are therefore exploring the use of additional building blocks, including D-amino acids, to modify peptides in order to create biostable peptide-like drugs (Muttenthaler *et al*., 2021). In a classical approach, a peptide made of natural-chirality L-amino acids is first developed using standard drug discovery techniques, and then modified to include non-canonical building blocks, including D-amino acids, to improve its stability while retaining its biological activity (Imanishi *et al*., 2021; Muttenthaler *et al*., 2021). For example, etelcalcetide, a drug for the treatment of secondary hyperparathyroidism, consists of a 7-amino acid D-peptide linked to a single L-amino acid (Blair, 2016).

Mirror-image proteins might also be used in drug discovery to aid the development of stable fully mirrored peptide drug candidates. Scientists can use standard drug discovery techniques to find natural-chirality peptides that bind to **mirror-image versions of a drug target**. By chiral symmetry, the mirror-image version of the peptide will then bind to the natural-chirality version of the target in patients, but is expected to have a significantly improved half-life because it would be resistant to protease degradation and immune recognition (Callahan *et al*., 2024; Harrison *et al*., 2023; Muttenthaler *et al*., 2021; Welch *et al*., 2010).

Several other applications of mirror-image proteins have been explored to date. For example, mirror-image proteins have been used to enable **protein structure determination** by X-ray crystallography. Chiral proteins can only form crystals in chiral space groups, whereas racemic mixtures can form centrosymmetric crystals, which significantly simplifies phase determination and thereby aids resolution of the protein structure (Mackay, 1989). This approach has been successfully used to determine a number of protein structures (Avital-Shmilovici *et al*., 2013; Banigan *et al*., 2010; Dang *et al*., 2016; Harrison *et al*., 2023; Huang *et al*., 2016; Hung *et al*., 1999; Luisier *et al*., 2010; Mandal, Pentelute, Tereshko, Kossiakoff *et al*., 2009; Mandal, Pentelute, Tereshko, Thammavongsa *et al*., 2009; Payne *et al*., 2021; Pentelute *et al*., 2010; Teng *et al*., 2021; Yeung *et al*., 2016).

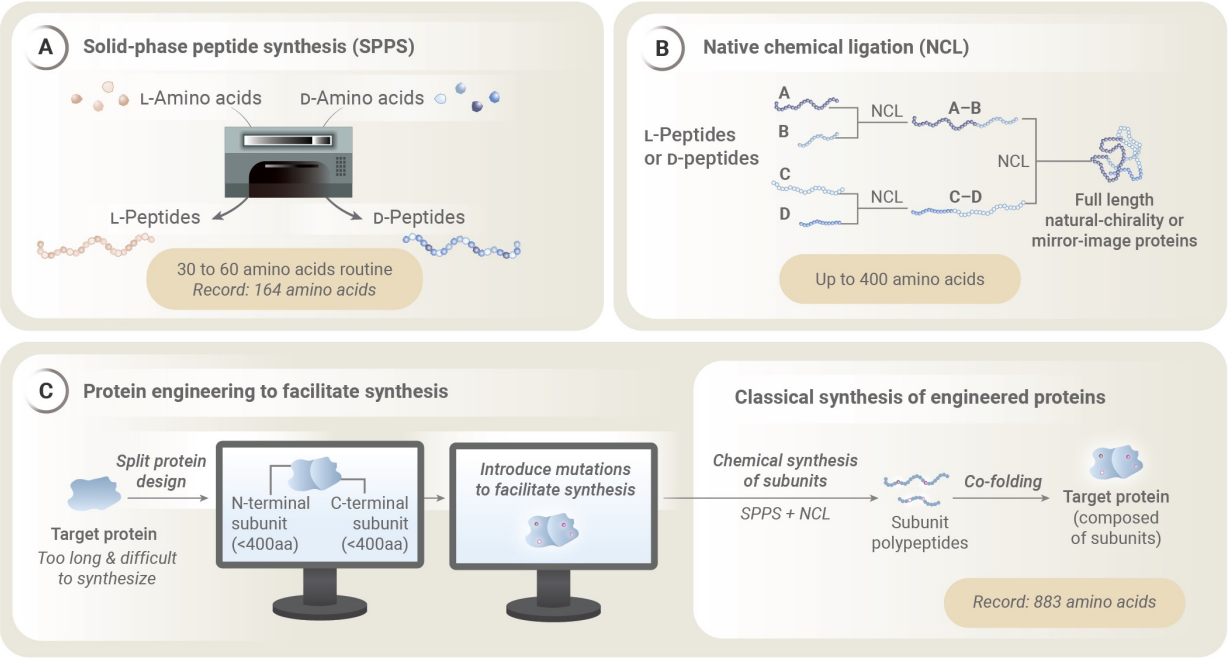

Figure 2.1: Chemical synthesis of mirror proteins
A. Solid-phase peptide synthesis can use L- or D-amino acid building blocks to produce L- or D-peptides. The process can be automated, and typically yields peptides of 30–60 amino acids in length, although longer chains can be achieved under optimized conditions. The current record is 164 amino acids (Hartrampf *et al.*, 2020). **B.** Native chemical ligation connects peptides irrespective of chirality to synthesize longer polypeptide chains. Applying this method in a hierarchical fashion, small to medium-sized proteins up to 400 amino acids can be produced. **C.** Protein engineering can facilitate the total chemical synthesis of functional equivalents of large proteins. In this approach, a target protein is split into multiple subunits, and mutations are introduced that facilitate synthesis while not affecting protein function. The subunits can then be made by a combination of solid-phase peptide synthesis and native chemical ligation, and the full protein yielded by co-folding the subunits. The current record is a triply split 883-amino acid protein (Y. Xu & Zhu, 2022).

the peptide segments produced in SPPS, and to introduce additional ligation sites to facilitate synthesis (Figure 2.1). The functionality of both split proteins was demonstrated, which enabled the transcription of kilobase mirror-image RNA, an important step toward the creation of mirror life.

As we will discuss in Section 2.2, the creation of a mirror bacterium through a bottom-up pathway would likely require the synthesis of at least 100 mirror proteins to create mirror ribosomes and other mirror transcription and translation machinery. Modern chemical protein synthesis technologies are in principle sufficiently advanced to synthesize most of the mirror proteins required. However, the cost and effort involved in synthesizing the large number of proteins required to create a cell bottom-up would be large. Scientists are continuously advancing methods for total protein synthesis that can be applied for mirror-image protein synthesis, including automated fast-flow peptide synthesis devices (Callahan *et al.*, 2024) or improved ligation methods (Agouridas *et al.*, 2019). Advances in protein engineering might likewise increase the ease and accessibility of split protein and mutagenesis approaches for chemical protein synthesis in the future (Dolberg *et al.*, 2021; Freschlin *et al.*, 2022; Lovelock *et al.*, 2022).

Alternatively, mirror protein synthesis might in the future be carried out using biological machinery, for example using *in vitro* ribosomal protein synthesis systems (Katoh & Suga, 2022). Unlike chemical approaches, biological machinery is inherently chirality-specific, so the production of mirror proteins using natural-chirality biological machinery is not feasible with current technology. Extensive academic research has explored re-engineering natural-chirality ribosomal protein synthesis systems to use building blocks other than canonical L-amino acids, including mirror D-amino acids (Katoh & Suga, 2022). A "crossover" translation system would use natural-chirality biological machinery to translate natural-chirality mRNAs into mirror-image proteins by polymerizing D-amino acids instead of natural L-amino acids. Notably, tRNAs charged with all 20 D-amino acids are available through *in vitro* flexizyme technology (Achenbach *et al*., 2015; Fujino *et al*., 2013). This crossover translation system is not necessary for the bottom-up creation of mirror cells if all the required components can be made by chemical synthesis. However, if an efficient and scalable biological mirror protein synthesis system were created in the future, it would be possible to rapidly synthesize most mirror proteins of interest. There is great interest in re-engineered protein translation systems for medical applications, hence progress in this area is expected even absent explicit interest in building mirror proteins (Katoh & Suga, 2022).

Mirror-image nucleic acids can be made chemically using established methods

Mirror-image DNA and RNA would be key components of a mirror cell. Current research, however, mainly focuses on potential applications as therapeutics or in biotechnology (see Box 2.2). DNA and RNA are chiral because the D-deoxyribose or D-ribose sugar backbone contains several chiral centers; the nucleobases are achiral. Methods to chemically synthesize the mirror-image L-ribose and corresponding mirror-image L-nucleotide building blocks of DNA and RNA have been available since the 1960s. Natural-chirality D-DNA oligonucleotides of up to 200 nucleotides and D-RNA oligos of up to 70 nucleotides are routinely produced by chemical solid-phase synthesis, a process that is highly automated and performed at scale by commercial DNA synthesis providers, and longer DNA or RNA oligos can often be produced, although at lower yields (Hoose *et al*., 2023). Adapting this chemical method for mirror DNA or RNA synthesis is straightforward. Several companies offer mirror DNA oligos commercially, although at higher prices due to the cost of mirror nucleotide building blocks (Biomers, n.d.; Glen Research, n.d.). Laboratories with DNA synthesis machines can also purchase mirror nucleotides directly and use them on existing machines to synthesize mirror oligonucleotides.

DNA oligos are commonly assembled into kilobase-length DNA pieces using enzymes such as DNA polymerase and DNA ligase (Hoose *et al*., 2023). RNA is made at kilobase length by transcription from a DNA template using RNA polymerase (Figure 2.2). The advances in chemical protein synthesis described in the previous subsection have recently enabled researchers to create mirror versions of these enzymes (Fan *et al*., 2021; Weidmann *et al*., 2019), permitting the assembly and

Box 2.2: Applications of mirror nucleic acids

DNA is commonly synthesized for recombinant DNA technology, but nucleic acids have numerous additional applications in basic science, biotechnology, and medicine. A drawback of DNA and RNA in these applications is their limited biostability, as they are recognized and degraded by biological processes. Mirror DNA and RNA retain properties of DNA and RNA, including the ability to form predictable structures determined by base-pairing, but are more stable as they are not susceptible to degradation by nucleases (enzymes which break down DNA and RNA) which are highly abundant in the environment.

These results led to interest in using mirror oligonucleotides as aptamer therapeutics and in other applications. **Aptamers** are oligonucleotides that can bind with high affinity to some biological molecule, e.g., a drug target. Aptamers can be discovered rapidly through directed evolution (Tuerk & Gold, 1990). However, similar to natural-chirality peptides, natural-chirality oligonucleotides are generally too unstable in physiological environments to be useful as aptamer drugs. In contrast, mirror-image aptamers—termed Spiegelmers by TME Pharma, the company developing them—could retain the binding properties but exhibit greater biostability and thus become useful drugs. Two Spiegelmers have reached early clinical testing as of 2024 (Kaur *et al*., 2018).

Mirror oligonucleotides might have diverse applications beyond aptamer therapeutics. For example, **molecular beacon** or **riboswitch** nucleic acids have been explored as biosensors (Seeman & Sleiman, 2017), and mirror-image versions of these can recapitulate their key properties while being more stable. Mirror image DNA nanostructures are being explored as drug delivery systems due to their greater resistance to serum host nucleases (Kim *et al*., 2016). They could also serve as interesting materials in **DNA nanotechnology** due to their ability to form predictable shapes with other mirror nucleic acids without interfering with natural-chirality nucleic acid assemblies (C. Lin *et al*., 2009).

accurate replication of kilobase-length mirror DNA, and the synthesis of kilobase-length mirror RNA from a corresponding mirror DNA template (Y. Xu & Zhu, 2022).

While synthetic mirror enzymes are currently available in only a handful of laboratories, current methods could in principle generate the mirror-image DNA genes required to assemble a mirror bacterial genome, each of which could be transcribed into mirror mRNAs, tRNAs, and rRNAs. However, assembling these genes into complete mirror chromosomes would be a major challenge, which will be discussed in Section 2.2.

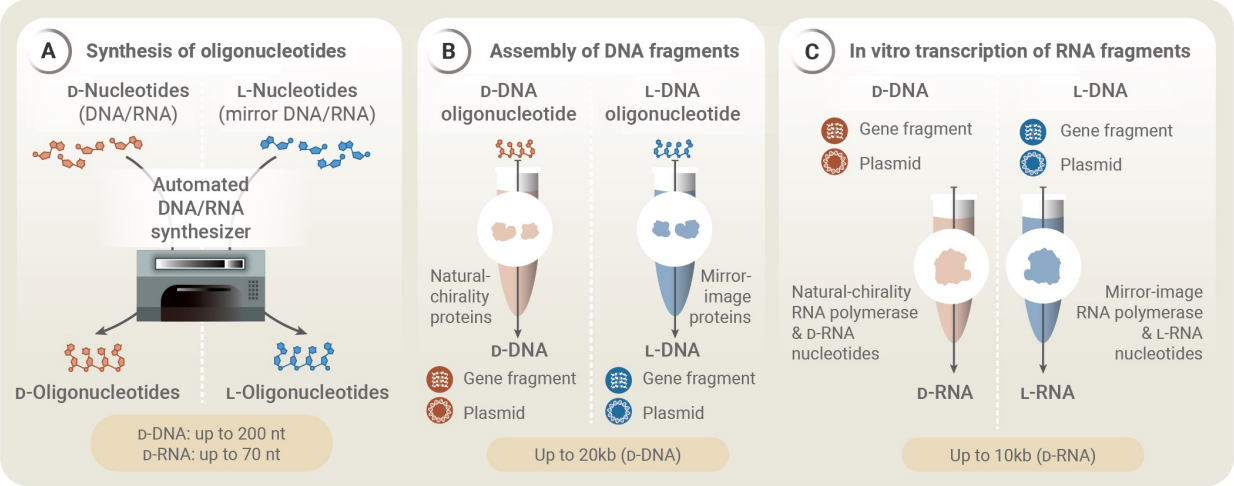

Figure 2.2: Synthesis of kilobase-length natural-chirality and mirror DNA and RNA
A. Synthesis of oligonucleotides from nucleotide building blocks by solid-phase synthesis can use D- or L-nucleotide building blocks to produce D- or L-oligonucleotides. This process is usually highly automated and typically yields DNA oligonucleotides up to 200 bp in length or RNA oligonucleotides up to 70 nucleotides in length. **B.** *In vitro* enzymatic reactions such as polymerase chain reaction and related methods can be used to assemble D-nucleotides into kilobase-length, double-stranded D-DNA constructs such as plasmids or gene fragments. Natural-chirality L-enzymes selectively act on natural-chirality D-DNA oligonucleotides, but these methods can be adapted for mirror L-DNA by using mirror-image D-proteins. **C.** *In vitro* transcription with a natural-chirality RNA polymerase can be used to produce kilobase-length D-RNA from a kilobase-length D-DNA template. This method can be adapted to use a mirror-image RNA polymerase to produce kilobase-length L-RNA from a kilobase-length L-DNA template.

2.2 Progress in synthetic biology could allow the assembly of a mirror bacterium from non-living mirror components

The ability to create a mirror bacterial cell could arise from the convergence of two distinct ongoing research programs. The first is the ability to synthesize the mirror-image versions of the necessary components of a bacterial cell, as described in Section 2.1. The second is ongoing research to reconstitute a living natural-chirality bacterium from synthetic components, which we will discuss in this section. Once the ability to do this is refined using normal-chirality molecules, it would become possible to repeat this process with mirror image components to create a living mirror bacterium.

This “bottom-up” pathway could involve the following steps:

1. Synthesis of a mirror genome *in vitro*.
2. Creation of mirror ribosomes and a high-efficiency *in vitro* mirror protein synthesis system from mirror components.
3. Encapsulation of the mirror genome and mirror protein synthesis system into a membrane and “booting” of the mirror bacterium.

We begin by providing a brief overview of existing synthetic cell research, then consider how each of the above steps could be accomplished with mirror-image components in the future.

Research is progressing toward the creation of self-replicating cells from non-living components

Bottom-up synthetic biology research attempts to build lifelike self-replicating cells from simple, non-living components. These components could be similar to those used in natural life—that is, be made of biomolecules like DNA, lipids, and proteins—or could instead use alternative chemistries to recapitulate the key processes of living cells.

A key feature of all cells is the presence of a lipid cell membrane that separates the cell interior from the environment. Synthetic lipid-bound compartments or vesicles were first created in the 1960s (Bangham *et al*., 1965; Chang, 1964), and diverse methods for the creation of vesicles have been developed since (Has & Sunthar, 2020). The lipid bilayer of natural cells consists in large part of phospholipids, and these can be used to create synthetic vesicles, but other lipid molecules can be used as well.

Gene expression, i.e., the synthesis of RNAs and proteins, is another key feature of living cells. Gene expression can be recapitulated in a test tube. For example, cell lysate maintains some transcription and translation activity if suitably prepared, and its productivity can be enhanced by providing an energy regeneration system. These so-called cell-free protein synthesis systems have found diverse applications for rapid prototyping, cell-free devices, rapid small-scale biomanufacturing, and beyond (Silverman *et al*., 2020).

Transcription and translation can also be accomplished using a fully defined system, known as "protein synthesis using recombinant elements" (PURE), in which the RNAs and proteins that make up the transcription and translation machinery (e.g., the RNA polymerase, ribosome, aminoacyl-tRNA synthetases, tRNAs, and translation factors) have been individually prepared and purified (Shimizu *et al*., 2001). This system has the advantage that its composition, unlike that of cell extract-based systems, is fully known and can be precisely controlled by including or omitting specific proteins or RNAs of interest. The drawback is that the PURE system is more expensive to prepare than lysate-based systems as it requires the preparation of a large number of recombinant proteins. Current PURE preparations are also generally less efficient at protein synthesis than extract-derived systems (Gregorio *et al*., 2019; Li *et al*., 2017).

DNA and transcription-translation systems can be encapsulated in lipid vesicles, creating artificial cells that resemble natural cells in that they are membrane-bound compartments capable of gene expression. Interestingly, membrane encapsulation can improve the protein synthesis efficiency of lysate-based systems (Garenne *et al*., 2021; Noireaux & Libchaber, 2004). Encapsulated transcription-translation systems can be used to produce diverse proteins, including metabolic enzymes or membrane proteins that are inserted into the vesicle membrane (Kuruma & Ueda, 2015).

The full central dogma—DNA replication, transcription, and translation—has recently been reconstituted within an artificial compartment (van Nies *et al*., 2018). Researchers expressed the DNA replication machinery from Φ29 phage within PURE-containing lipid vesicles and showed that the encoding DNA could subsequently be replicated. The advantage of a Φ29 phage replication system is its comparative simplicity; but recapitulating the DNA replication machinery from bacteria such as *E. coli* in a lipid vesicle would likely be of interest to researchers in the future, as it might

permit the replication of synthetic genomes greater than the ~150 kb size limit of the Φ29 replication machinery.

Ongoing research aims to encode the PURE components within the encapsulated genome to create a self-sustaining central dogma. In one attempt, a 116 kb genome encompassing the full set of *E. coli* central dogma machinery was encapsulated in an artificial compartment; however, it was not possible to achieve PURE synthesis at self-sustaining levels, or ribosome biogenesis (Libicher *et al*., 2020). Future work on enhancing PURE synthesis efficiency, and work aimed at improving *in vitro* ribosome biogenesis, might allow scientists to accomplish this milestone in the future (Hagino & Ichihashi, 2023; Lavickova *et al*., 2020; Wei & Endy, 2021).

Alternatively, researchers might attempt to encapsulate a full bacterial genome within a vesicle containing transcription and translation machinery in order to "boot" a live bacterium. In one attempt, researchers encapsulated the *E. coli* genome and a lysate-derived transcription-translation solution in a synthetic lipid vesicle and observed the expression of a reporter gene from the genome (Deyama *et al*., 2021). However, achieving a full "boot", i.e., the creation of a self-replicating bacterium, might require better transcription-translation efficiencies, encapsulation methods that ensure the genome is not damaged by shearing forces, and the use of native phospholipid membrane (unlike the artificial lipid membrane used in Deyama *et al.*). As noted above, improved transcription-translation systems now produce almost as much protein as the input protein required to create the transcription-translation solution (Garenne *et al*., 2021), and such high-efficiency transcription-translation systems have enabled the boot of a 40 kb T7 phage genome within a lipid vesicle to generate functional T7 phage (Garenne *et al*., 2021; Shin *et al*., 2012). The boot of a live bacterium from a cell-derived genome, transcription-translation system, and membrane thus appears increasingly feasible—several of the authors are pursuing optimized strategies and are aware of colleagues doing the same.

Bottom-up synthetic cell research is progressing rapidly, and ever more complex behaviors of cells, including movement, sensing and response to external stimuli, cell-cell communication, synthetic organelles, and multicellular assemblies have been reconstituted with synthetic cells (Rothschild *et al*., 2024). This research is already leading to interesting applications (Adamala *et al*., 2024). Liposomes play a key role in the delivery of mRNA therapeutics and gene therapies, and scientists are exploring artificial liposomes as therapeutic delivery devices. While most hallmarks of life have been reconstituted in artificial cell-like structures in isolation (Gaut & Adamala, 2021), a frontier of current research is to integrate the different systems in a single self-replicating cell to create an artificial compartment that exhibits all features of life—that is, to create life in the test tube (Kriebisch *et al*., 2024).

The synthesis of a mirror genome *in vitro* might be attainable by building on existing technology

To create a mirror bacterium, researchers would need to synthesize a mirror DNA genome. Chemical DNA oligo synthesis and enzymatic assembly, as discussed in Section 2.1, permits the synthesis of mirror DNA up to several kilobases in length. The synthesis of a mirror genome would require

large-scale synthesis of the genome in kilobase-length pieces by these methods, and their subsequent assembly into a megabase-length genome. The exact length would depend on the specific mirror bacterium to be created: the synthesis of a minimized *Mycoplasma mycoides* genome, the smallest genome known to be viable, would require a 0.53 megabase (Mb) genome (Hutchison *et al*., 2016) whereas the genome of *E. coli* strain K-12 MDS42, a common laboratory strain, would require 3.98 Mb (Pósfai *et al*., 2006).

Scientists have successfully synthesized a small number of natural-chirality bacterial genomes, but each synthesis still involves major effort and the *in vivo* replication of increasingly large pieces within bacteria or yeast (Fredens *et al*., 2019; Gibson *et al*., 2010). The first challenge in adapting these methods for the synthesis of a mirror genome is the cost of synthesizing the required DNA in kilobase-length pieces, which can be up to $1 million USD from commercial DNA synthesis providers for a large genome; the cost to synthesize an equivalent amount of mirror DNA could be orders of magnitude higher.

The second challenge is the assembly of the kilobase-length pieces into a megabase-scale genome. Larger-scale DNA assembly into 50–100 kb pieces, and their subsequent assembly into Mb-length pieces, is typically done in living yeast or *E. coli* (Fredens *et al*., 2019; Gibson *et al*., 2010). Such methods could not be adapted to assemble mirror DNA unless mirror organisms were already available. However, DNA pieces up to 900 kb in length can also be assembled *in vitro* using synthetic enzymes (Gibson *et al*., 2009). It appears likely that extensions of this or other methods could be used to assemble Mb-scale mirror DNA using mirror enzymes, although fidelity and yield may need to be optimized. Alternatively, it may be possible to boot synthetic cells with genomes composed of multiple small chromosomes similar to some existing bacterial strains (diCenzo & Finan, 2017; Itaya & Tanaka, 1997; K. Wang *et al*., 2019; Yoneji *et al*., 2021). Including fewer replication and assembly steps could reduce the opportunity for errors that could disrupt essential genes and potentially ease the process of encapsulating the DNA within a membrane.

Current *in vitro* DNA synthesis and assembly methods exhibit a low but non-negligible error rate, and genome synthesis projects typically use clonal amplification *in vivo* to propagate and verify assembly intermediates. However, methods such as digital PCR (Vogelstein & Kinzler, 1999) or clonal amplification in synthetic vesicles (Abil *et al*., 2023) could serve a similar purpose for an *in vitro* effort, and gene synthesis researchers have substantially improved *in vitro* error correction methods (Hoose *et al*., 2023; Sidore *et al*., 2020; J. Zhang *et al*., 2020).

Overall, while the creation of a synthetic genome entirely *in vitro* would require the adaptation and improvement of a number of existing assembly and error-correction methods to work with mirror DNA, the synthesis of which would be extremely expensive at current prices, this step does not appear to be an insurmountable bottleneck to the bottom-up creation of a mirror bacterium.

Research is progressing toward mirror ribosomes and a mirror protein synthesis system

Bacteria contain thousands of RNAs and proteins. However, it may not be necessary to create all cellular components from scratch to assemble a living cell. RNA synthesis can be accomplished by a single protein: RNA polymerase. In the PURE system mentioned above (Shimizu *et al*., 2001),

protein synthesis can be accomplished with as few as 36 proteins, ribosomes (comprising several RNAs and about 50 proteins), tRNAs, and a defined chemical energy mix.

If scientists were to succeed at creating sufficiently efficient mirror transcription and translation systems from a handful of mirror-image RNAs and mirror proteins, and combine them with a mirror genome, they could synthesize directly from the mirror genome the thousands of proteins and RNAs required for life. The resulting mirror proteins could then synthesize mirror lipids, carbohydrates, a cell wall, and metabolites. Hence, to generate mirror proteins outside of a cell, researchers would at least need to synthesize the mirror components of the minimal PURE translation system: mirror aminoacyl-tRNA synthetases, mirror translation factors, mirror tRNAs, and a mirror ribosome (Shimizu *et al*., 2001).

Ribosomes are complex structures consisting of several large rRNAs and many ribosomal proteins (r-proteins). For example, the *E. coli* ribosome consists of three rRNAs and 54 r-proteins (Figure 2.3). In the natural-chirality PURE system, ribosomes are typically purified from cells, which would not be possible for mirror ribosomes. Instead, mirror-image r-proteins and unmodified mirror rRNAs would need to be created chemically, and subsequently assembled into functional mirror-image ribosomes *in vitro*. It would be important to assemble intact ribosomes with productivities similar to native ribosomes, as the efficiency of the translation system is expected to be critical to successfully booting up a bacterium.

The assembly of small ribosomal subunits entirely from *in vitro*-generated rRNA and protein has not yet been reported. However, small ribosomal subunits made from *in vitro*-generated rRNA and *in vivo*-derived r-proteins are functional (Krzyzosiak *et al*., 1987), suggesting that small subunit rRNA modifications are not essential for function. Making mirror small ribosomal subunits therefore seems feasible after some optimization. The construction of large ribosomal subunits from *in vitro*-generated rRNA and protein has likewise not been achieved. Functional ribosomal large subunits can be made from *in vitro*-generated rRNA and *in vivo*-derived purified r-proteins, but at substantially reduced activity (Aoyama *et al*., 2022; Semrad & Green, 2002). Ribosomal large subunits with greater activity can be generated in extract-based systems, which may contain enzymes that supply at least some of the post-transcriptional modifications and other proteins that aid ribosome biogenesis (Jewett *et al*., 2013). Identifying these rRNA modification enzymes and ribosome biogenesis factors might therefore enable the *in vitro* synthesis of large ribosomal subunits. Making mirror large ribosomal subunits that support protein synthesis with high efficiency thus seems like a difficult but tractable technical step.

Native tRNAs, like rRNAs, are extensively modified by post-transcriptional modification enzymes in bacteria like *E. coli*, and *in vitro*-produced tRNAs and rRNAs lack such modifications. Experimental work shows that these modifications are essential to achieve high translation activity for the tRNAs corresponding to 3 out of 20 amino acids (Cui *et al*., 2015) so creating mirror-image versions of these tRNA-modifying enzymes might be necessary to achieve high mirror PURE productivity. However, it has been shown that a complete reconstitution of *in vitro*-produced tRNAs lacking chemical modifications could function in the PURE system when their concentrations are optimized. Therefore, synthetic mirror tRNAs could function in the mirror PURE system (Iwane *et al*., 2016; Iwane *et al*., 2018). As noted in a previous section, the PURE system can produce most proteins,

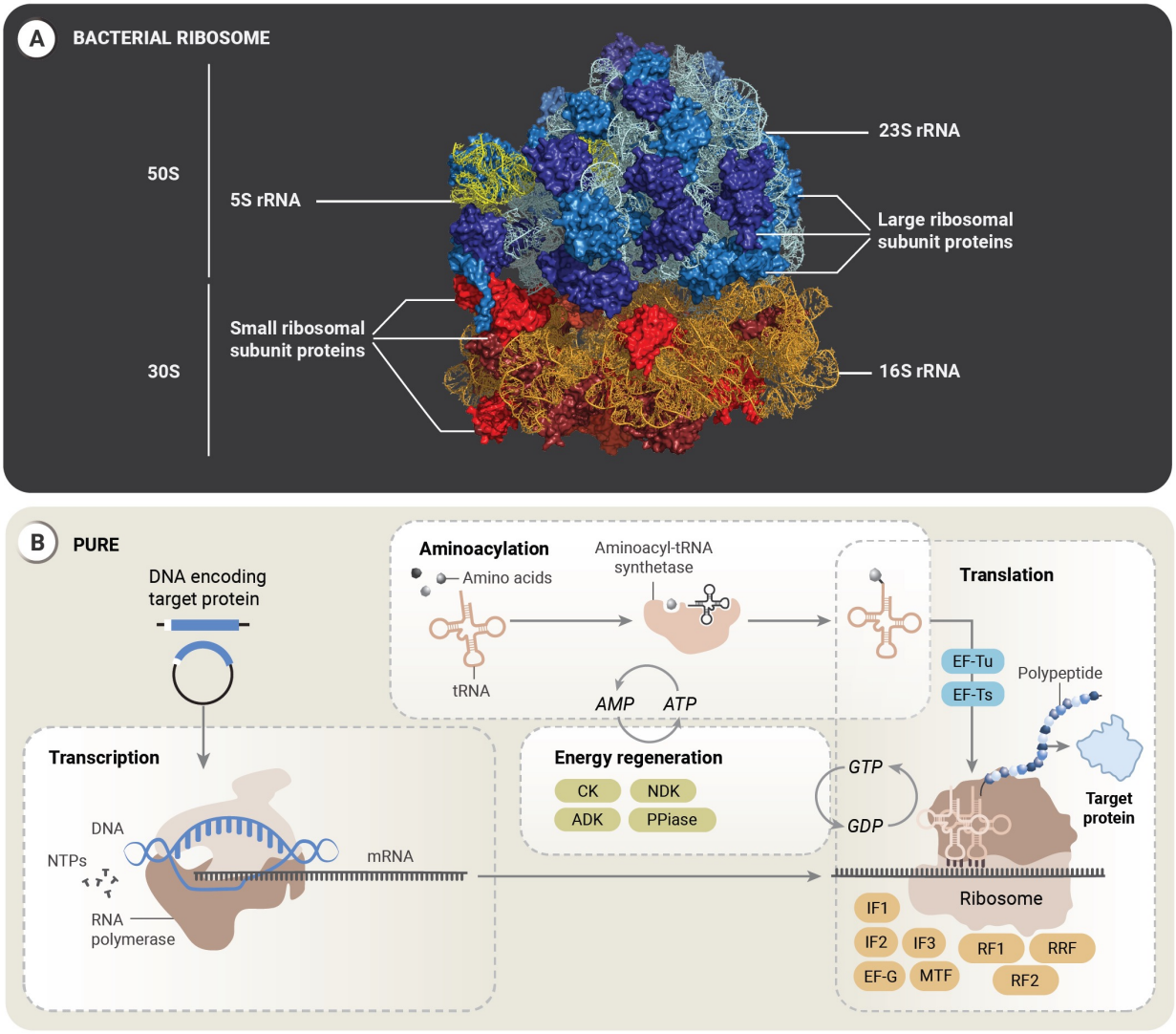

Figure 2.3: Components of ribosomes and the PURE system
A. Cryo-EM structure of the *E. coli* ribosome (PDB 5AFI). The *E. coli* ribosome consists of two major subunits, the 50S large subunit and the 30S small subunit. The 50S subunit consists of two rRNAs (23S, light blue, and 5S, yellow) and 34 r-proteins (blue and dark blue). The small subunit consists of one rRNA (16S, orange), and 21 r-proteins (red and dark red). **B.** Components of the PURE system. DNA encoding a target protein is added to the PURE system, which consists of all proteins, amino acids, nucleotides, and non-coding RNAs necessary to transcribe it into mRNA and translate the mRNA into the desired polypeptide.

including membrane proteins (Kuruma & Ueda, 2015), but PURE synthesis generally yields lower amounts of protein product than a comparable amount of cellular protein synthesis machinery, and below self-sustaining levels. Therefore, the synthesis capacity of current PURE systems is unlikely to be sufficient to produce a cellular proteome as part of an attempt to "boot up" a living cell from a synthetic genome.

Several research groups are actively working to improve the PURE system's efficiency, with the aim of developing a PURE system with the capacity for self-regeneration (Hagino & Ichihashi, 2023;

Lavickova *et al*., 2020; Wei & Endy, 2021). Challenges for the original PURE formulation might include lack of important translation factors, chaperones and supporting proteins, lack of homeostasis, and non-native protein concentrations (about 10-fold lower than in the cytosol). Research approaches to increase PURE's productivity therefore include improved systems to provide energy during the reaction and remove byproducts, altering the concentration of the components, and adding additional protein factors (de Maddalena *et al*., 2016; Kazuta *et al*., 2014; Lavickova *et al*., 2020; Li *et al*., 2017). Progress in this field would likely be required to permit the booting experiments described in the following subsection. However, it is difficult to determine precisely what level of PURE efficiency would be required. A fully self-regenerating PURE system might not be necessary; during the boot, the PURE system might produce the missing translation factors, chaperones, and other supportive proteins.

Encapsulating the mirror genome and mirror protein synthesis system in a membrane could "boot up" the mirror bacterium

If mirror proteins and nucleic acids were successfully used to create a mirror genome and a mirror protein synthesis system *in vitro*, the final step to creating a mirror bacterium would involve combining those components in a lipid compartment and attempting to boot up a living, self-replicating cell.

The lipids in the bacterial membrane are chiral. Bacterial membranes consist primarily of phospholipids with a chiral L-glycerol-3-phosphate backbone. Notably, archaeal membranes consist primarily of phospholipids with a D-glycerol-3-phosphate (more commonly known as L-glycerol-1-phosphate) backbone alongside other differences, and experiments have shown that *E. coli* can grow without fitness defect with 20–30% archaeal phospholipid content in its membrane (Caforio *et al*., 2018). In any case, mirror lipids can be synthesized chemically, and so are unlikely to be a bottleneck for mirror bacterium assembly.

The bacterial cell wall is likewise chiral, and the chemical construction of its mirror-image appears difficult. However, the construction of a mirror cell wall may not be necessary. Some bacterial species, most notably *Mycoplasma,* naturally lack a cell wall. *E. coli* has a cell wall, but experiments have shown that *E. coli* can replicate without a cell wall and subsequently regenerate it (Petrovic Fabijan *et al*., 2022; Tabata *et al*., 2019). Therefore, scientists could plausibly create mirror bacteria without chemically constructing a cell wall.

Scientists have not yet created a living cell of any chirality in a test tube from non-living components. As discussed above, a first milestone might be the assembly of a living natural-chirality cell in a test tube from cell-derived components (Figure 2.4A). In this experiment, the genome, cell lysate/cytosol, and cell membrane would first be harvested from living bacteria, and then recombined to create a living cell. This experiment could establish the core methods for combining a genome, protein synthesis system, and membrane in a way suitable to boot a live bacterium. It is plausible that such an experiment will succeed in the next few years with moderate improvements to existing experimental protocols, for instance by improving the efficiency of extract-based transcription-translation systems and gentle encapsulation methods that would prevent shearing of

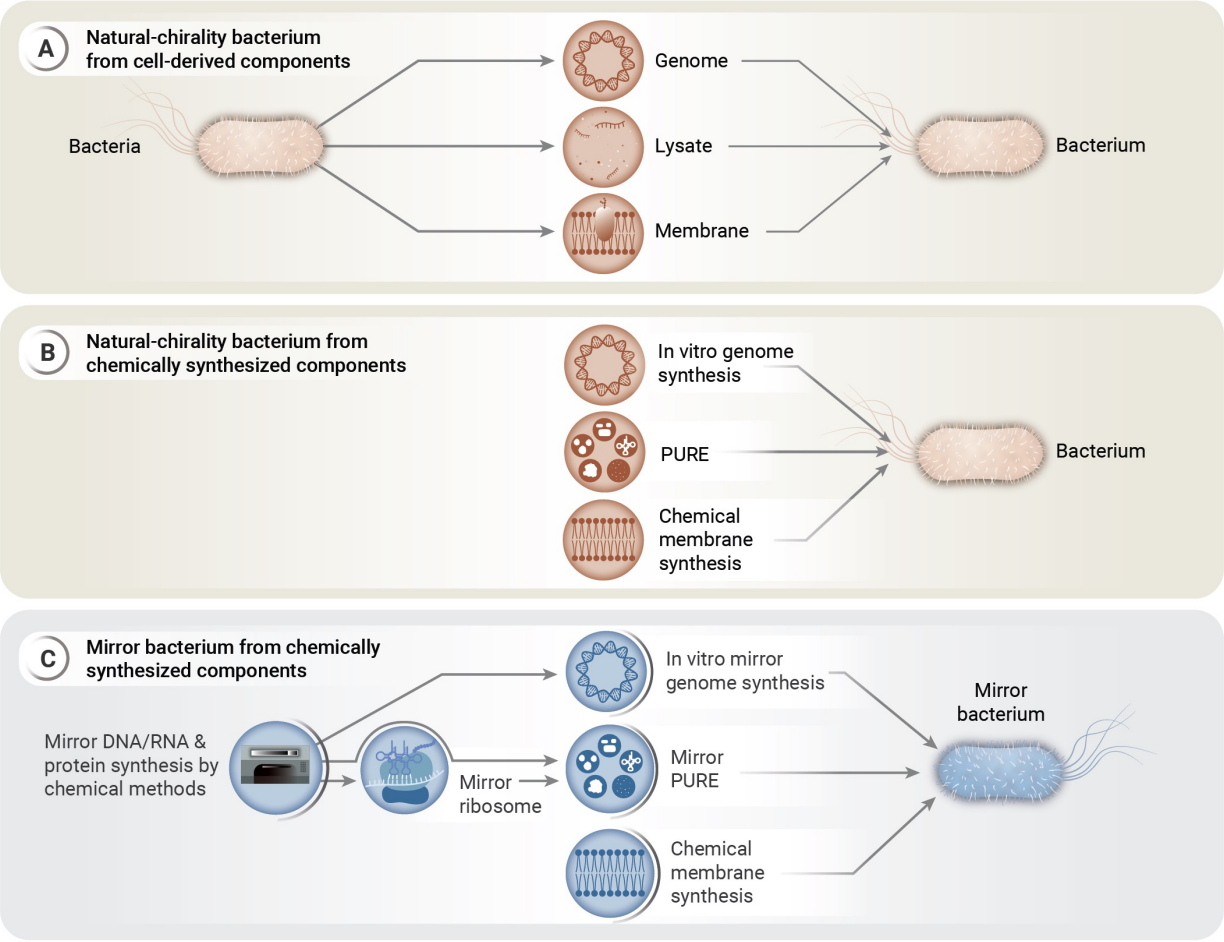

Figure 2.4: Milestones towards the creation of a mirror bacterium
A. Schematic depicting the boot of a living natural-chirality bacterium from non-living, cell-derived components. In this experiment, the bacterial genome, lysate, and membrane would be harvested from an existing bacterium and then recombined to create a new living bacterium. **B.** Schematic depicting the boot of a living natural-chirality bacterium from non-living, fully synthetic components. In this experiment, the genome, lysate, and membrane from A would be replaced by synthetic components made without the help of existing cells, such as a synthetic genome, the PURE system, and synthetic lipids. **C.** Schematic depicting the boot of a mirror bacterium from non-living, fully synthetic mirror components. In this experiment, mirror versions of the components in B would be made and then combined using the protocol established in B to boot a mirror bacterium.

the genome. However, it may be necessary to extensively optimize the preparation of the individual components, the genome, and the booting-up protocol to create a living cell. The difficulty of this step is hard to predict, but the necessary technologies for such an attempt are available to scientists today.

Each of the three components in the cell reconstitution—genome, cell lysate, and membrane—would need to be replaced with a fully synthetic version (not one purified from other cells) to subsequently enable the creation of a mirror bacterium using the same protocol (Figure 2.4B).

Ideally, the starting PURE system would have sufficient synthesis capacity to generate the full proteome from the genome and thereby boot a self-replicating cell in a single step. As noted in the

previous section, the required synthesis efficiency is hard to determine, but it might be less than the synthesis capacity needed for self-regeneration, as there might be positive feedback in the expression of the genome due to the production of translation factors, chaperones, and other auxiliary systems that might help to facilitate further genome expression. However, should this fail, it may be possible to do a "partial" boot, recover the newly synthesized partial proteome (e.g., by removing the "used-up" PURE system using affinity tags and concentrating the new proteins), and add the new components to another booting attempt. Iterations of this process might generate a large fraction of the proteome, enhancing the PURE synthesis capacity until it crosses the threshold for positive feedback to make the entire proteome.

Positive feedback is not guaranteed, however. Some intermediate booting states could be toxic to the cell or the booting process (e.g., translation of proteases, nucleases, or membrane channels before their regulators). If the source of failure were found, it might be addressed by supplementing specific proteins to the original PURE mixture or making changes to the booting genome; the viability of the altered genome could be confirmed in separate experiments. Advances in creating cells with minimal genomes might be informative and minimal cells might be easier to boot, as their genomes are less complex (Haimovich *et al*., 2015; Hutchison *et al*., 2016). Alternatively, advanced genome design or powerful whole-cell models could be used to simplify or otherwise optimize the target genome and hence reduce the complexity of the booting task.

Overall, a boot from fully synthetic components would be a difficult technical step in the bottom-up pathway. However, as research in bottom-up synthetic biology progresses, the enabling methods will continue to improve. The exact timelines and challenges are hard to forecast with precision, but as synthetic cell research progresses rapidly, the natural-chirality version of this booting experiment might well succeed within the coming decade (Adamala *et al*., 2024). Once this has been accomplished, it might "simply" be a matter of investing the resources required to produce mirror versions of all the components, as outlined in the previous sections, and then applying the booting protocol to produce a synthetic mirror bacterium (Figure 2.4C).

2.3 A natural-chirality bacterium might be converted into a mirror bacterium in a stepwise fashion

As an alternative to the "bottom-up" path outlined in Section 2.2, a mirror bacterium could be created by a "top-down" pathway in which a living natural-chirality bacterium is converted into a mirror bacterium. This pathway might require more extensive technology development compared to the "bottom-up" pathway in Section 2.2, but these technologies are likely to benefit from greater investments for reasons unrelated to mirror life. A "top-down" strategy to create mirror bacteria might involve three steps:

1. Production of mirror-image proteins *in vivo* by creating a crossover pathway made of natural-chirality components.
2. Production of mirror-image proteins *in vivo* by creating an entirely mirror-image central dogma.

3. Delivery or assembly of a full mirror-image DNA genome *in vivo*, and removal of the natural-chirality genome, to create a mirror bacterium.

Such work would be able to build on existing research on genetic code reprogramming. We begin by outlining these existing approaches, and then consider each step in turn.

Genetic code reprogramming allows for the production of proteins with non-canonical amino acids

Research in genetic code expansion and reprogramming aims to create living cells that produce proteins made of an expanded set of amino acids. These non-canonical amino acids (ncAAs) are chemically diverse and thereby confer new properties to proteins. This includes a plethora of tools to study protein function and create enhanced proteins, such as ncAAs that crosslink to protein interaction partners, bioorthogonal reaction handles that allow site-specific protein modification, and mimics of post-translational modifications (Chin, 2017; Young & Schultz, 2018). More recent work has seen re-engineering of translation to create polymers made entirely of ncAAs, creating new classes of biopolymers that could serve as future materials or therapeutics (de la Torre & Chin, 2021).

The incorporation of an ncAA can be achieved by expressing a suitably engineered aminoacyl-tRNA synthetase/tRNA (aaRS/tRNA) pair in a cell (Mukai *et al*., 2017; Young & Schultz, 2018). The aaRS charges the ncAA onto its cognate tRNA, which then can be used by the ribosome to incorporate the ncAA. In many experiments, the tRNA is engineered to decode the TAG stop codon, as TAG-decoding tRNAs compete efficiently with release factor 1 to permit site-specific incorporation of the ncAA at the TAG codon (Mukai *et al*., 2017).

An important requirement is that the aaRS/tRNA pair needs to be orthogonal to the host translation machinery; that is, the additional aaRS must charge only its cognate tRNA and none of the host tRNAs, and the additional tRNA likewise must not be charged by any of the host aaRSs. Moreover, the aaRS active site must only charge the ncAA of interest onto its tRNA and not any of the canonical amino acids or other small molecules present in the cell.

Building on these advances, in recent years researchers have been exploring ways of using multiple ncAAs in parallel in the same cell, toward creating a translation pathway for the synthesis of genetically encoded non-canonical biopolymers (de la Torre & Chin, 2021). There are three principal requirements for such a pathway:

1. Blank codons that can be used to encode additional ncAAs need to be created.
2. Mutually orthogonal aaRS/tRNA pairs that can work in parallel with the host aaRS/tRNA pairs and each other and can charge multiple distinct ncAAs need to be found.
3. If ncAAs with complex side-chains or altered backbone structures are to be used, the ribosome and other translation factors might need to be engineered to enable their efficient polymerization.

Researchers have been progressing rapidly on these three challenges in recent years (de la Torre & Chin, 2021). For example, there are multiple pathways that might create blank codons in cells. The

genetic code consists of 64 three-letter codons encoding 21 signals, the 20 naturally occurring amino acids and a "stop" or termination signal. Many codons are synonymous; they encode the same signal. Synonymous recoding of a genome involves choosing a particular codon, and substituting all instances of that codon with a synonymous codon throughout the genome. The recoded codon no longer appears in the genome. Its cognate tRNA or release factor may be deleted, or reassigned to a new, potentially non-canonical, amino acid.

The falling cost of DNA synthesis and progress in synthetic genomics has recently enabled the synthesis of codon-compressed genomes of this kind. In 2013, the first such codon-compressed genome was developed by multisite editing (Lajoie *et al*., 2013; H. H. Wang *et al*., 2009). The TAG stop codon was replaced by synonymous alternatives genome-wide, which allowed the deletion of release factor 1 and hence reassignment of TAG to ncAAs at enhanced efficiency (Lajoie *et al*., 2013). Subsequently, researchers created an *E. coli* with a fully synthetic genome containing just 61 codons, in which TAG as well as TCG and TCA, two of the six serine codons, were removed and substituted with synonymous alternatives (Fredens *et al*., 2019). This organism, dubbed Syn61, contains three blank codons that can be reassigned to three non-canonical amino acids and used to synthesize short non-canonical polymers (Robertson *et al*., 2021).

Genome recoding is not necessary if blank codons can be created by a different pathway. In one strategy, scientists engineered a ribosome to read quadruplet (4-base) codons rather than canonical triplet (3-base) codons (Neumann *et al*., 2010). In principle this provides up to 256 blank codons, and researchers have shown that up to four non-canonical amino acids can be used in parallel in response to four quadruplet codons (Dunkelmann *et al*., 2021). However, quadruplet decoding suffers from competition with triplet decoding in these experiments, limiting the efficiency of ncAA incorporation. Combining recoding with quadruplet decoding can address this challenge (Chatterjee *et al*., 2014). Codon context is another strategy that has been used to optimize quadruplet decoding (Costello *et al*., 2024).

In an alternative approach, researchers have created an organism that uses an additional nucleotide pair alongside the A:T and C:G pair that makes up canonical DNA. The additional pair (dTPT3:dNaM) can be replicated as part of the DNA, transcribed into RNA, and used to encode protein synthesis in cells (Malyshev *et al*., 2014; Y. Zhang *et al*., 2017). Future progress in creating an expanded genetic alphabet that can be used efficiently to code for non-canonical amino acids might likewise provide a diverse range of blank codons in the future. In *in vitro* experiments, researchers have also considered the possibility of creating parallel genetic codes, where one type of ribosome translates the proteome using the canonical genetic code, and a different type of ribosome uses a separate tRNA pool with a different assignment between triplet codons and amino acids (Terasaka *et al*., 2014).

The development of mutually orthogonal aaRS/tRNA pairs is challenging due to the numerous orthogonality requirements. Much work in the field has been driven by the discovery that *Methanocaldococcus jannaschii* tyrosine-tRNA synthetase (TyrRS)/tRNA pair and the pyrrolysyl-tRNA synthetase (PylRS)/tRNA pairs from *Methanosarcina mazei* and *Methanosarcina barkeri* fulfill these orthogonality requirements *in vivo* and that their active sites can be engineered to charge a diversity of ncAAs with large hydrophobic and aromatic side chains (Mukai *et al*., 2017).

Nonetheless, several additional pairs beyond the *M. jannaschii* TyrRS/*M. mazei* and *M. barkeri/*PylRS pairs have recently been found by exploring the natural sequence diversity of synthetases across the kingdoms of life. Up to five of these pairs have been found to be mutually orthogonal (Beattie *et al*., 2023). Most pairs are derived from tyrosine and pyrrolysine synthetases, and hence available ncAAs mostly have large hydrophobic or aromatic side chains and a canonical α-L-amino acid backbone. However, further effort at discovering more diverse aaRS/tRNA pairs and developing improved methods for their engineering will likely increase the number and diversity of ncAAs that can be used in coming years.

Most approaches in genetic code expansion rely on the promiscuity of the ribosome. However, while the ribosome is generally permissive to different side chains, the efficiency of ncAAs with altered backbone structures (such as N-alkyl, D-, or β-amino acids) is generally reduced (de la Torre & Chin, 2021). Ribosome engineering is challenging due to the central role of ribosomes in metabolism, and many active site mutations are lethal. However, researchers have laid the foundation for ribosome engineering by creating so-called orthogonal ribosomes (Orelle *et al*., 2015; Rackham & Chin, 2005; Schmied *et al*., 2018). These are copies of the ribosome that are isolated from host translation and translate their own set of orthogonal mRNAs, which are not read by the host ribosome (Figure 2.5). Initial experiments indicate that such ribosomes can be engineered to use some challenging amino acids with increased efficiency (Schmied *et al*., 2018).

Extensions of genetic code reprogramming might permit the creation of a crossover pathway for *in vivo* mirror protein synthesis made of natural-chirality components

Researchers studying genetic code expansion and reprogramming are re-engineering protein translation to use non-canonical amino acids, including D-amino acids (Katoh, Iwane *et al*., 2017), in addition to the canonical amino acids. Extensions of this approach could in principle enable the creation of a crossover translation path for the production of mirror proteins inside natural-chirality bacteria (Figure 2.6A–B). Advancing this technology to enable *in vivo* mirror protein synthesis would likely require:

1. Generating 19 blank codons in the *E. coli* genome, one for each D-amino acid needed.
2. Generating 19 orthogonal aaRS/tRNA pairs that charge the 19 D-amino acids onto orthogonal tRNAs decoding the blank codons.
3. Engineering a crossover ribosome that can efficiently polymerize D-amino acids, and perhaps engineering other supporting translation factors.

As discussed in the previous section, many methods for generating blank codons are being studied, but synonymous codon compression is one possible approach. Synthesizing a viable genome that uses only 45 codons could free up 19 blank codons. A challenge in creating codon-compressed genomes is that synonymous codons can have coding functions in addition to specifying the order of amino acids, such that synonymous codon replacements can have fitness costs to the bacterium or be lethal. The codon replacement scheme in the 61-codon Syn61 genome was determined by empirical testing, and the final organism grew 60% slower than its parent 64-codon organism, although some

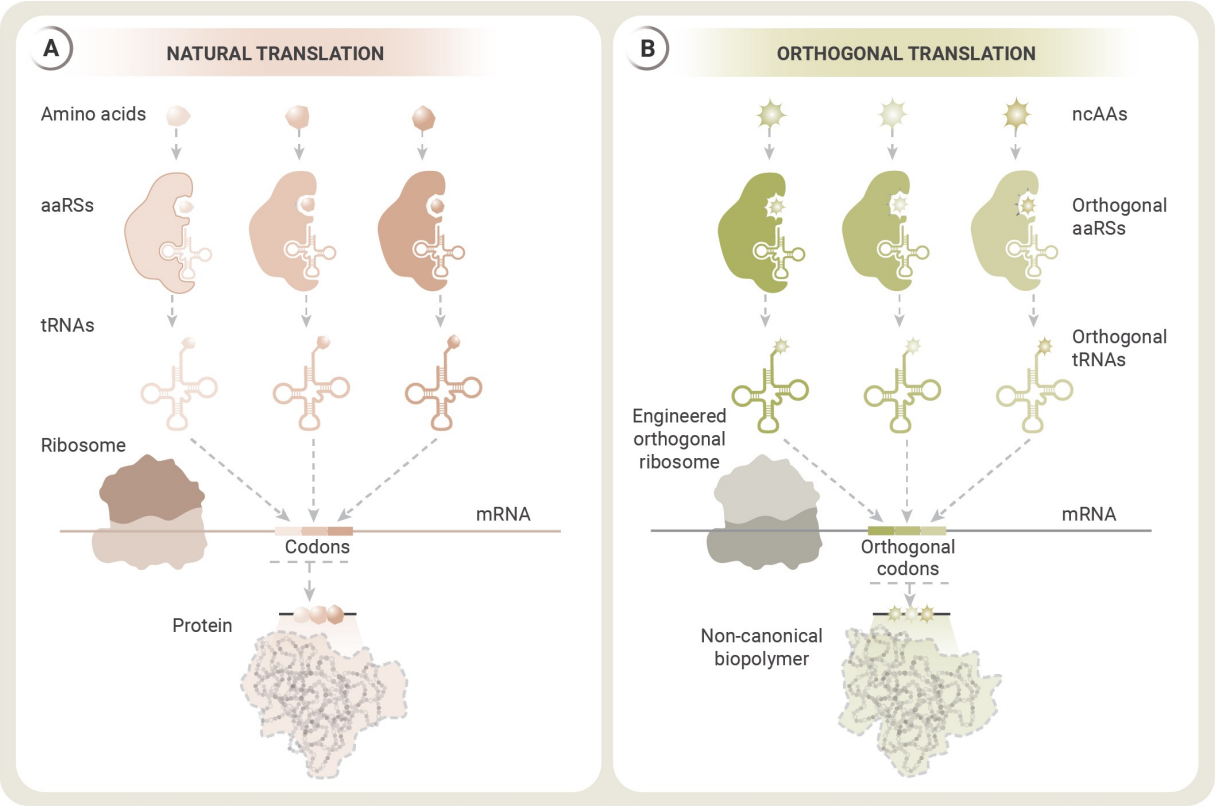

Figure 2.5: Genetic code reprogramming allows the genetically encoded synthesis of non-canonical biopolymers in cells
A. Natural translation. Aminoacyl-tRNA synthetases acylate cognate tRNAs with amino acids, which are then used by the ribosome to make a protein. **B.** Orthogonal translation. The genetic code is reprogrammed to enable engineered orthogonal aminoacyl-tRNA synthetases to acylate orthogonal tRNAs with non-canonical amino acids (ncAAs). The resulting aminoacyl-tRNAs can then be used to decode orthogonal codons, potentially by an evolved orthogonal ribosome, to produce non-canonical biopolymers.

of that fitness loss could be recovered by subsequent laboratory evolution (Fredens *et al*., 2019; Robertson *et al*., 2021).

A 45-codon organism would still have substantial redundancy in its genome; a hypothetical "minimal-code" genome would only use 21 codons (for the 20 canonical amino acids and stop codon). As our understanding of synonymous codon choices and predictive and empirical methods to design recoded genomes advances, the creation of such a codon-compressed genome might become increasingly feasible.

Codon compression is just one strategy to generate the required blank codons. Alternatively, if the efficiency of quadruplet decoding or of an expanded genetic alphabet could be improved, D-amino acids could be encoded by these newly generated codons. Likewise, if a parallel translation pathway in which a separate ribosome selectively uses its own pool of tRNAs *in vivo* could be constructed, codons could be selectively reassigned in this parallel translation pathway without interfering with cellular translation. While substantial progress will be required to generate 19 blank codons by any of

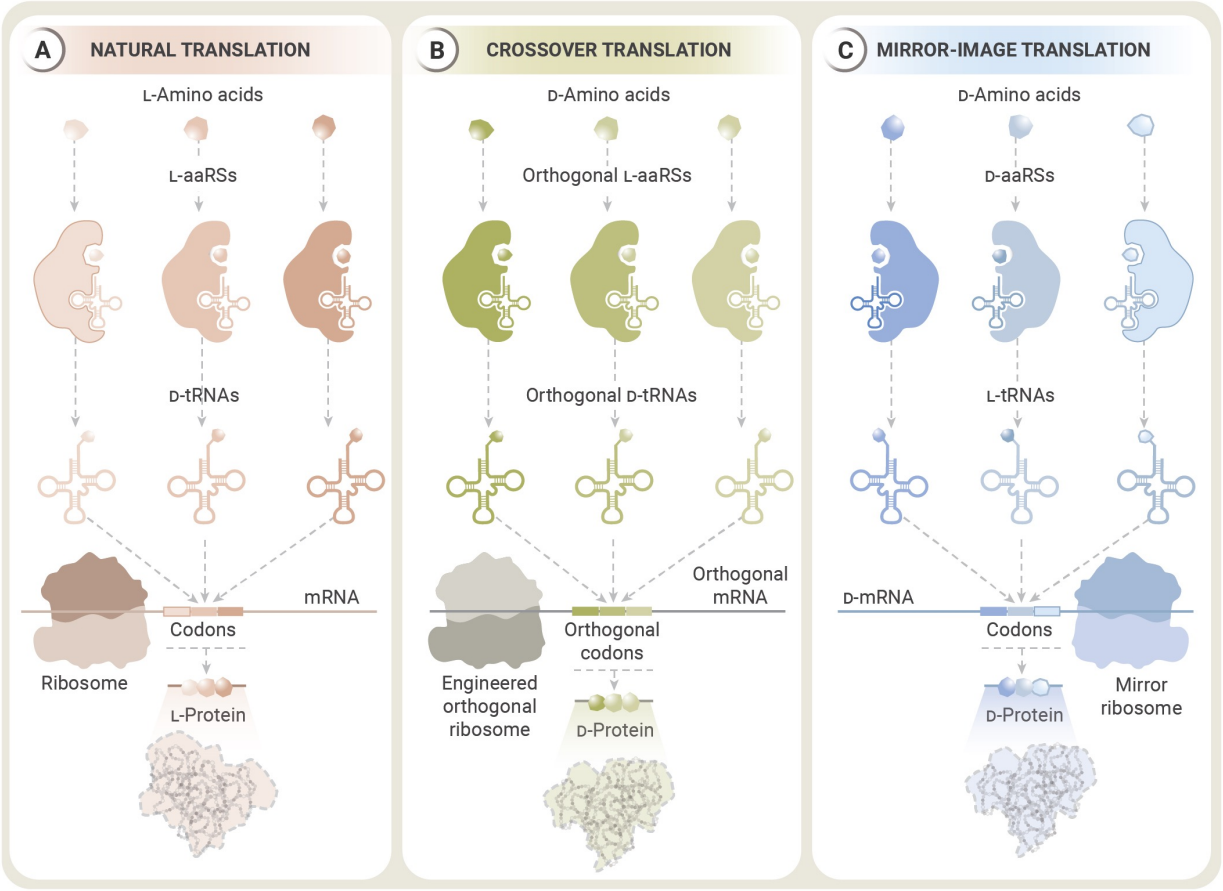

Figure 2.6: Natural, crossover, and mirror-image translation
A. Natural translation. L-Amino acids are charged by L-aminoacyl-tRNA synthetases onto D-tRNAs, which read codons. L-Amino acids are polymerized by a natural-chirality ribosome to produce L-proteins. **B.** Crossover translation. D-Amino acids are charged by orthogonal L-aminoacyl-tRNA synthetases onto orthogonal D-tRNAs, which read orthogonal codons. D-Amino acids are polymerized by an engineered orthogonal natural-chirality ribosome to produce D-proteins. **C.** Mirror-image translation. D-Amino acids are charged by D-aminoacyl-tRNA synthetases onto L-tRNAs which read codons on a D-mRNA. D-Amino acids are polymerized by a mirror ribosome to produce D-proteins.

these approaches, and further technical hurdles such as wobble decoding would need to be addressed, it seems likely that this will eventually be possible.

The *in vivo* incorporation of D-amino acids would require the generation of 19 aaRS/tRNA pairs that could load the 19 D-amino acids onto tRNAs to be used for *in vivo* mirror protein synthesis. Each aaRS/tRNA pair would need to be orthogonal in its aaRS/tRNA recognition to other aaRS/tRNA pairs used for D-amino acid incorporation and the host aaRS/tRNA pairs that use the canonical L-amino acids, exclusively and efficiently charge their cognate D-amino acid and not other D- or L-amino acids, and exclusively and efficiently decode their target codon.

While no aaRS/tRNA pairs for D-amino acid incorporation *in vivo* have yet been reported, and creating pairs that fulfill these requirements would be a major protein engineering effort, most of the necessary methods have been described in principle. This includes approaches for discovering

additional orthogonal aaRS/tRNA pairs (Cervettini *et al*., 2020), improving their efficiency (Amiram *et al*., 2015), and methods for engineering synthetases to charge non-canonical amino acids with altered backbones (Dunkelmann *et al*., 2024). Further advances in this field or in related areas, e.g., computational methods for protein design, would lower the expertise and resource requirements for this task further.

Genetic code expansion has so far mostly relied on the promiscuity of the ribosome toward non-canonical amino acids. However, while the ribosome is generally permissive toward non-canonical amino acids with altered side chains, amino acids with altered backbones such as D-amino acids are polymerized less efficiently. *In vitro,* under highly optimized conditions, up to ten D-amino acids can be polymerized consecutively by the ribosome*,* but at significantly reduced efficiency compared to L-amino acids (Katoh, Tajima, & Suga, 2017). Key translation factors such as EF-Tu, which delivers aminoacyl-tRNAs to the ribosome, are also specific to the L-amino acid backbone (Arranz-Gibert *et al*., 2018). Hence, it is likely that it would be necessary to engineer the ribosome and translation factors to support more efficient polymerization of D-amino acids *in vivo* to enable the cellular synthesis of mirror proteins. It is an open question to what extent ribosomal mutations can be found that enhance D-amino acid incorporation *in vivo*, and it is hard to predict how efficient the crossover ribosomes would be. However, as tools for ribosome engineering continue to be developed (d'Aquino *et al*., 2018; de la Torre & Chin, 2021) and general protein engineering and directed evolution methods improve (Simon *et al*., 2019; Yang *et al*., 2019), this challenge is becoming increasingly tractable. For example, a ribosome engineered to incorporate D-amino acids and not L-amino acids, with a Shine-Dalgarno sequence that is orthogonal to the existing ribosome, would minimize the risk of unwanted crosstalk between normal and mirror protein creation (Dunkelmann *et al*., 2021; Rackham & Chin, 2005).

The levels of D-amino acid required for efficient mirror protein translation from a crossover ribosome might be toxic to existing cells (Aliashkevich *et al*., 2018). Many D-amino acids are already present in existing organisms, though at lower concentrations than L-amino acids. Extracellular concentrations in the millimolar range of certain amino acids, especially of D-Trp, can inhibit growth due to misincorporation by the ribosome (Leiman *et al*., 2013; Rumbo *et al*., 2016; Soutourina *et al*., 2000; see also Box 1.2); intracellular concentrations of the corresponding L-amino acid enantiomers are only a few-fold lower. That is, the current canonical tRNA/aaRS pairs for existing L-amino acids may misincorporate D-amino acids too frequently to tolerate the higher concentrations that would be needed to support a crossover ribosome. The enzyme D-aminoacyl tRNA deacylase (DTD) functions to hydrolyze tRNAs mischarged with D-amino acids. DTD is highly promiscuous, functioning on a wide range of D-amino acids and an equally broad range of tRNA structures, which may make it difficult to produce orthogonal tRNAs intended to be charged with D-amino acids by their own aaRS enzymes. Deleting DTD would resolve this problem, but doing so greatly increases the toxicity of D-amino acids to bacteria (Leiman *et al*., 2013). In principle, DTD and the new tRNAs might be engineered to be orthogonal to one another. Alternatively, artificial organelles might be used to sequester the mirror components, including D-amino acids, away from the rest of the cell; this possibility is discussed further in Section 2.4.

A crossover pathway could be used to create a self-sustaining mirror central dogma *in vivo*

Once mirror proteins could be made *in vivo* using the crossover pathway, the next step would involve creating pathways for the production of mirror DNA and RNA *in vivo* (Figure 2.6C). Mirror DNA could be replicated *in vivo* by making mirror versions of the enzymes that replicate DNA, i.e., initiator proteins, a DNA polymerase, helicase, ligase, topoisomerase, single-strand binding protein, and other accessory proteins that make up the replisome (Oakley, 2019). Additionally, it would be necessary to provide a pathway for the synthesis of mirror-image nucleotide triphosphate (NTP) building blocks. Once these mirror proteins were produced inside the cell, it might be possible to transform an *in vitro*-made mirror DNA plasmid into the cell, which would then be propagated by the mirror replisome. Once mirror DNA could be propagated within a bacterium, RNA could be produced by expressing a mirror RNA polymerase.

In the crossover pathway, a natural-chirality protein synthesis system is used to create mirror proteins (Figure 2.6B). However, once both mirror proteins and mirror RNA can be produced *in vivo*, scientists might be able to construct a mirror version of translation (Figure 2.6C). Unlike the crossover pathway, a mirror version of translation would use mirror translation machinery made of mirror proteins and mirror RNA to create mirror proteins. Once this is established, it could be self-sustaining: mirror replication, transcription, and translation machinery could be encoded in mirror DNA, which is then transcribed and translated by the mirror machinery. At this point, the crossover translation system could be removed. This would yield a mixed chirality bacterium that simultaneously carried a natural-chirality genome and a natural-chirality proteome similar to natural life, but additionally carried a mirror DNA plasmid and mirror central dogma machinery capable of creating mirror DNA, RNA, and proteins (Figure 2.6C).

Establishing a mirror central dogma might face some technical challenges. For example, the synthesis of mirror ribosomal RNA and mirror ribosomal proteins might have to be coordinated for *in vivo* mirror ribosome biogenesis, and suitable mirror RNA-modifying enzymes would additionally need to be present. The many components of the mirror translation system might interfere with the natural-chirality host cellular machinery in hard-to-predict ways. Nonetheless, while troubleshooting this system at this stage would require skillful experimentation, it would not necessarily require the large resource investments that are involved in creating 19 blank codons or engineering 19 mutually orthogonal aaRS/tRNA pairs, for example, or the synthesis of a large number of mirror proteins like in the bottom-up approach.

A mirror bacterium could potentially be created by delivering a mirror DNA genome and removing the natural-chirality genome

Finally, researchers might expand the mirror DNA plasmid to encompass a complete mirror genome and remove the natural-chirality genome from the cell, to create a fully mirrored bacterium (Figure 2.7). One strategy could be to build a mirror genome *in vitro* and deliver it to the mixed-chirality cell in a single step. If the mirror genome is taken up, the mirror transcription and translation machinery could begin gene expression from the mirror genome, producing all the mirror proteins and RNAs

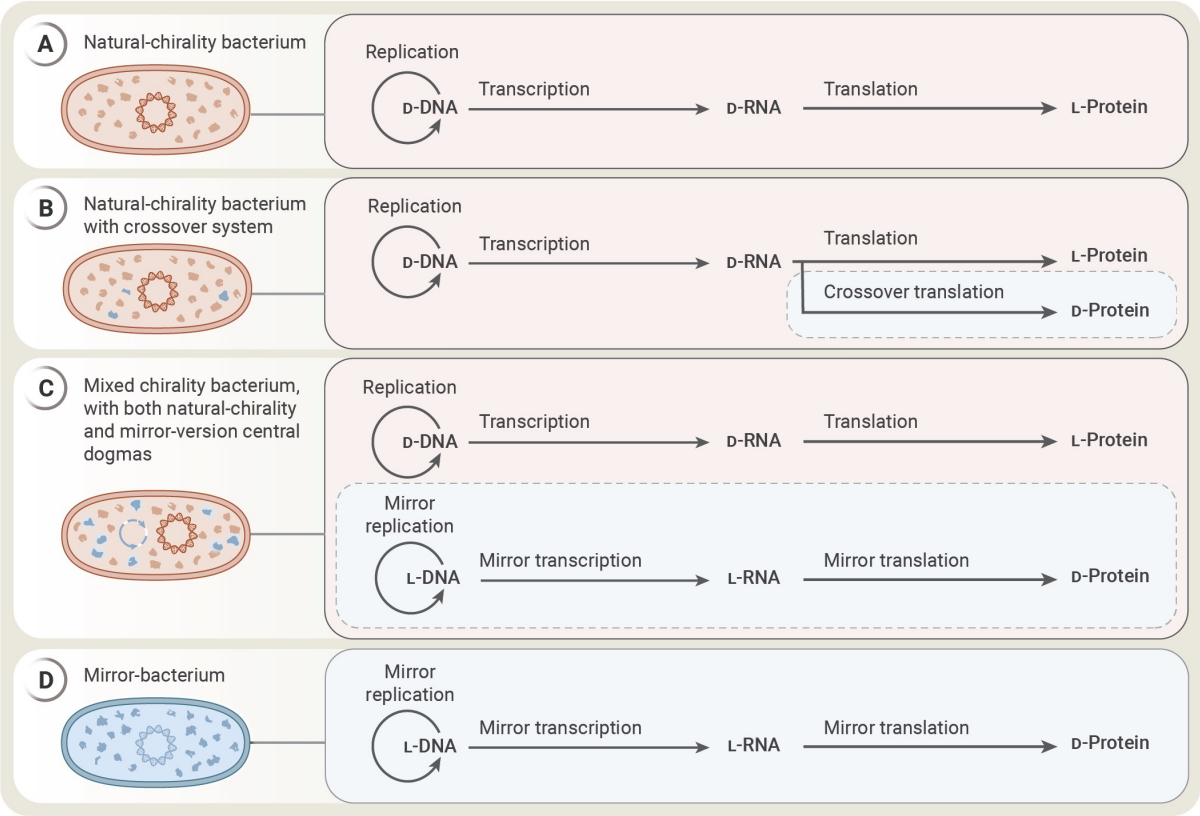

Figure 2.7: Stepwise conversion of a natural-chirality bacterium into a mirror bacterium
A. Stage 1: A natural-chirality bacterium with a natural-chirality central dogma. **B.** Stage II: A natural-chirality bacterium that additionally contains a crossover translation system made of natural-chirality components that is able to produce mirror proteins *in vivo*. **C.** Stage III: A mixed-chirality bacterium that contains a natural-chirality central dogma and a mirror central dogma. **D.** Stage IV: A fully mirrored bacterium that contains a mirror genome and a mirror central dogma and no natural-chirality components.

that make up a mirror bacterium. At the same time, enzymes that degrade the natural-chirality genome could be expressed, thereby stopping gene expression from the natural-chirality genome. Hence, after some turnover, the mirror genome, transcriptome, and proteome would be expressed whereas the natural-chirality genome, transcriptome, and proteome would be degraded. If the transition can be successfully managed, the result would be a viable mirror cell. This is conceptually similar to the "booting" experiment in the bottom-up pathway.

This chirality conversion step could be made less drastic, improving the chance of success, by expanding the DNA episome in a stepwise fashion to build a mirror genome. A challenge in this approach is that the co-expression of some natural-chirality and mirror genes could cause toxicity as some mirror proteins would likely interfere with regular cellular processes. For example, mirror bacterial cell division proteins might interfere with cell division, or mirror metabolic enzymes with metabolism. To mitigate this, researchers could try to replace sets of proteins governing natural-chirality functional modules, such as cell division or energy metabolism, entirely with mirror components. This would result in a step-by-step conversion of natural-chirality to mirror bacteria in which some functional modules would be natural-chirality, and some mirror, during the conversion

stages. Like the previous step, the cell conversion step would be a technically challenging but tractable path to create a mirror bacterium.

2.4 Other approaches to creating mirror bacteria are plausible

Science rarely proceeds in linear fashion, and progress is unlikely to precisely follow the pathways outlined in the above sections. In this section, we turn to consider some pathways that appear more speculative based on our current understanding of the challenges, but could become more plausible in the coming decades as related scientific fields advance.

Research along the bottom-up and top-down pathways would not proceed in isolation, and would be able to draw on wider progress in the synthetic cell, genetic code expansion, and bioproduction communities. For example, an *in vivo* system for the synthesis of mirror proteins could provide mirror proteins for a bottom-up approach. Similarly, *in vitro* methods for the synthesis of a mirror DNA genome would facilitate the introduction of a mirror DNA genome in a natural-chirality cell. Insights as to the difficulties of booting up a genome in the bottom-up approach might inform the boot in a top-down path, and vice versa.

As discussed in Section 2.2, there is great academic interest in generating synthetic cells from non-living components. The first synthetic cell could be a replica of an existing organism and use DNA, proteins, carbohydrates, and other macromolecules familiar from natural life. However, there is also work aimed at creating synthetic cells from abiological components, for example using compartments other than phospholipid vesicles (Discher *et al*., 1999; Holowka *et al*., 2005; Joesaar *et al*., 2019; Shang *et al*., 2022; van Swaay *et al*., 2015) or atypical or abiological macromolecules to perform key cellular functions (Jiang *et al*., 2022; Zhan *et al*., 2022). Similarly, researchers are interested in synthesizing plausible early forms of life to study the origin of life, for example RNA-only protocells (Joyce & Szostak, 2018). Most artificial cells of this kind are unlikely to be viable outside the laboratory. However, the creation of such artificial cells may facilitate the creation of a mirror bacterium by stepwise conversion of the abiological artificial cell into a mirror bacterium.

Establishing a crossover and a mirror translation system inside a living bacterium, as discussed in Section 2.3, would be challenging and labor-intensive. An alternative approach could be to temporarily and fully switch the protein synthesis machinery from natural-chirality protein production to mirror protein production. If this could be done, the natural-chirality DNA genome could be temporarily translated into a mirror proteome. With the mirror proteome in place, if a mirror DNA genome could be delivered at this stage and the natural-chirality DNA genome degraded, it might be possible to rapidly convert a natural-chirality bacterium into a mirror bacterium. This strategy is attractive as it might be easier to temporarily remodel the translation machinery for mirror protein synthesis using the natural-chirality genome than to stably co-express mirror protein synthesis systems alongside the natural-chirality protein synthesis that keeps the natural-chirality bacterium alive. However, there would be fewer opportunities for troubleshooting compared to the path outlined in Section 2.3.

Another modification of the approach outlined in Section 2.3 would employ a natural organelle like the mitochondrion (which is non-essential for yeast replication) or the creation of an artificial

organelle inside a natural-chirality organism to avoid the need for creating blank codons and to sequester the potentially toxic mirror components. Researchers have successfully constructed artificial organelles made of proteins and of lipid membranes in bacteria using a variety of methods and used them to isolate metabolic pathways from the rest of the cell (Oerlemans *et al*., 2021). For example, introducing peroxisomal proteins from eukarya into bacteria has generated membrane-encapsulated artificial peroxisomes (Cross *et al*., 2017). The same peptide tags used to transport peroxisomal enzymes in eukaryotes (Baker *et al*., 2024) can direct tagged proteins and associated biomolecules into the bacterial peroxisomes, allowing them to perform biochemical reactions in isolation. Bacterial organelles harboring independently replicating DNA exist in nature: the bacterium *Candidatus Thiomargarita magnifica* features numerous membrane-bound organelles called pepins that contain copies of the genome and/or active ribosomes (Volland *et al*., 2022). It appears likely that the organelles themselves replicate independently, much like mitochondria in eukaryotic cells. Such an organelle could in principle harbor an independent episome encoding the desired mirror proteins, the polymerase required to transcribe them, and the crossover translational machinery—including D-amino acids at high concentrations—capable of producing them *in vivo*. As artificial organelle as well as mitochondrial and chloroplast engineering advance, it appears plausible that the technologies required to sequester a mirror central dogma within a natural-chirality cell could be developed.

Future advances in enzyme engineering could enable the creation of enzymes using energy to drive the conversion of L-amino acids to D-amino acids within a polypeptide chain. A “protein handedness converter”, if it could be created, could provide a shortcut to create mirror life: it could be used to produce mirror-image proteins *in vitro* for the bottom-up pathway, or *in vivo* for a top-down pathway, or enable the creation of a transient crossover translation system as discussed above.

In recent years, progress in artificial intelligence (AI)-based scientific methods have had a transformative impact in the life sciences. It is plausible that further advances in AI might accelerate solutions for many of the technical hurdles in the approaches outlined above. This could include designing enzymes to manipulate mirror biomolecules, strategies to create key components such as synthetic ribosomes, engineered translational machinery to facilitate *in vivo* mirror protein production, or improved genome design approaches that might facilitate booting or cell conversion experiments, among others. AI might also help to accelerate experimentation, for example by facilitating laboratory automation.

2.5 The feasibility of mirror life will increase as related technologies advance

In the previous sections, we found that multiple plausible pathways to the creation of mirror bacteria exist, although major technological hurdles remain.

In the bottom-up pathway outlined in Section 2.2, the most notable bottlenecks are the development of recipes for the assembly of self-replicating cells from a protein synthesis system, a membrane, and a genome, alongside strategies for creating entirely synthetic ribosomes. Moreover, advances that would reduce the cost and effort of mirror protein and nucleic acid synthesis would greatly facilitate

any effort toward the creation of a mirror bacterium. Finally, existing genome synthesis approaches would need to be adapted to enable the synthesis of a mirror genome without *in vivo* manipulations.

In the cell conversion pathway described in Section 2.3, a major bottleneck is the development of a crossover translation pathway that would allow the synthesis of mirror proteins entirely with natural-chirality cellular machinery. This, in turn, would require the creation of 19+ blank codons that could be assigned to D-amino acids or an organelle system for sequestration, alongside the development of suitable aaRS/tRNA pairs that could use the 19 D-amino acids *in vivo*, and a crossover ribosome capable of polymerizing these D-amino acids efficiently. Moreover, extensive cellular engineering may be required to maintain a mirror central dogma within a natural-chirality cell, and to facilitate the final cell conversion step to create a fully mirrored bacterium.

Overall, our analysis shows that the creation of a mirror bacterium is not possible with current technology absent extensive investment of time and resources to overcome several scientifically challenging and labor-intensive technical hurdles. However, we found no technical hurdle that would pose a fundamental obstacle to the creation of a mirror cell, and we expect that dedicated research programs could eventually resolve each bottleneck.

While few laboratories explicitly focus on the creation of mirror life, many of the enabling technologies are actively being developed by a large community of academic and industrial researchers for reasons unrelated to mirror life. For example, a large community of researchers is pursuing synthetic cell research for applications in basic science and medicine without the aim of creating a mirror bacterium; likewise, researchers in genetic code reprogramming are advancing methods toward the *in vivo* production of non-canonical biopolymers without an explicit interest in mirror bacteria. For these reasons, we expect that over time, with scientific progress, many of the technical hurdles will be overcome even in the absence of explicit efforts to create mirror life. This will increasingly open up pathways to the creation of mirror bacteria. While forecasting technological progress is highly uncertain, absent governance of the enabling technologies, we estimate that mirror bacteria could be created within the next 15–30 years; or sooner, if substantial resources were invested in a focused effort.

Chapter 3: Engineering, Biosafety, and Biosecurity of Mirror Bacteria

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As outlined in subsequent chapters, due to their opposite chirality, sufficiently robust mirror bacteria could plausibly evade immune responses and resist predation, allowing them to infect an unusually wide range of multicellular organisms and invade diverse environments. Depending on the path through which they are created, the first mirror cells might not be very robust—perhaps more akin to a synthetic minimal cell than a natural bacterium. However, it would likely be relatively straightforward to convert a fragile mirror bacterium into the mirror image of a robustly growing natural-chirality bacterium, yielding a robust mirror bacterium. This distinguishes mirror bacteria from other types of highly engineered bacteria or synthetic cells, where the creation of fast- and robust-growing strains is often challenging. Though biocontainment methods could reduce risks from mirror bacteria, these safeguards are susceptible to human error or could be undone through genetic engineering. Because fragile and biocontained mirror bacteria could be converted into more dangerous versions by a nefarious or reckless actor, the creation of any mirror bacterium would create a major security risk.

In Section 3.1, we discuss how any mirror bacterium could be used as a starting point to generate additional mirror bacterial strains and species, and how these mirror bacteria could be augmented using routine genetic engineering. While creating the first mirror bacterium would be a major undertaking, subsequent modifications could be accomplished using standard genetic engineering techniques. Using these methods, a fragile mirror bacterium could be converted into a strain that grows robustly and further enhanced—for example, by engineering its ability to metabolize natural-chirality nutrients.

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In Section 3.2, we discuss how physical and biological biocontainment approaches might reduce the risk of an inadvertent release of mirror bacteria. Making the growth and survival of mirror bacteria dependent upon multiple molecules not found in nature would reduce the chance of an accidental release. Given the serious potential harms of such a release, however, the safety standards for mirror bacteria would need to be substantially greater than those applied to natural-chirality organisms.

In Section 3.3, we consider the biosecurity implications of mirror bacteria. We find that, while the initial creation of a mirror bacterium would be technically challenging, the conversion of a fragile or biocontained mirror bacterium into a potentially dangerous mirror bacterium could be accomplished with relative ease, posing serious security concerns.

3.1 The creation of any mirror bacterium could enable the generation of diverse mirror bacterial strains and species and their modification by routine genetic engineering

As outlined in Chapter 2, the creation of a mirror bacterium would be a highly challenging undertaking. Depending on its path to construction, the first mirror bacterium might be intrinsically fragile, metabolically limited, and hampered by genetic artifacts of the assembly process. Such an organism would likely have insufficient robustness and fitness for invasion of the environment and might require numerous mirror-image nutrients that are rare or absent in nature. However, compared with the initial creation of a mirror bacterium, the transformation of such a bacterium into more robust mirror bacterial strains or species would be notably less challenging. In addition, mirror bacteria could be augmented with new capabilities through genetic engineering, including the ability to digest natural-chirality sugars.

Theoretical applications of mirror bacteria in industry and medicine (Box 3.1) would require robustly growing mirror bacterial strains and would benefit from strains capable of digesting natural-chirality nutrients. Absent concerns regarding potential safety and security risks, these applications would create incentives for the development of such mirror bacteria.

Existing techniques for engineering bacteria could be adapted to mirror bacteria

Because mirror biology and natural-chirality biology would behave symmetrically, most tools and techniques developed for genetic engineering of natural-chirality bacteria could be adapted for mirror bacteria without further development or requirements beyond access to synthetic mirror DNA, the mirror bacterium of interest, and a small set of mirror protein reagents for more complex manipulations. While accessibility decreases as the required changes become more numerous and complex, simple changes—such as providing antibiotic resistance and engineering a mirror bacterium to feed on natural-chirality nutrients like D-glucose—would be straightforward for individuals with basic molecular biology training.

Scientists have been genetically engineering bacteria for decades and have developed powerful tools for doing so. Notable successes include the production of recombinant proteins such as insulin (Goeddel *et al*., 1979; Overton, 2014), the production of valuable small molecules by fermentation

Box 3.1: Some potential applications of mirror bacteria would require high-risk strains

The initial development of mirror bacteria may be driven by academic interest rather than interest in any specific application. Nevertheless, mirror bacteria could have potential applications in both industry and medicine if not precluded by the serious biosafety and biosecurity risks outlined in this report.

Mirror bacteria could be used to **manufacture mirror biomolecules** such as mirror-image nucleic acids or mirror-image proteins. These mirror biomolecules can have applications in drug discovery and beyond, as discussed in Chapter 2. Mirror bacteria would provide a straightforward route to manufacturing these molecules at scale, similar to the production of recombinant natural-chirality DNA or natural-chirality proteins in natural-chirality bacteria. This would require the creation of a mirror bacterium (like *E. coli* or *B. subtilis*) that grows robustly and is fit enough to pay the metabolic costs of biomolecule production. Such strains would benefit from being able to metabolize natural-chirality nutrients such as D-glucose, as growth on natural-chirality media would be substantially cheaper than creating synthetic media with mirror nutrients. Hence, even if biocontained through other means, such bioproduction strains would be closely related to mirror bacterial species that could potentially act as pathogens and spread uncontrollably in the environment.

There are several alternatives to mirror bacterium-based manufacture of mirror biomolecules. Mirror biomolecules can be made at small to medium scale using existing chemical synthesis approaches (Harrison *et al*., 2023), and future research might improve the accessibility, scale, and cost-effectiveness of these methods (Tan *et al*., 2020). In the future, scientists could develop methods for mirror biomolecule synthesis using *in vitro* enzymatic approaches (using natural-chirality or mirror enzymes) that do not rely on self-replicating cells (Weidmann *et al*., 2019). Scientists might also succeed in engineering natural-chirality bacteria to produce mirror biomolecules (de la Torre & Chin, 2021).

More speculatively, mirror bacteria might be engineered to act as **immune-invisible live cell therapeutics.** The increasing ability to genetically engineer bacteria has led to increased interest in using bacteria as live 'smart' therapeutics that could seek out a specific environment within a human patient, sense the disease state, compute an appropriate response, and produce effector molecules correspondingly to treat the disease (Riglar & Silver, 2018). Mirror bacteria might present an interesting chassis for live cell therapeutics, as they are expected to have greatly reduced recognition by mammalian immune systems (see Chapter 4). If growth of the mirror bacteria could be made dependent on one or several unnatural metabolites—i.e., a synthetic auxotrophy—their growth within a human host could be precisely controlled (Mandell *et al*., 2015). However, therapeutic applications are generally incompatible with physical containment or access control. A therapeutic mirror bacterial strain would need to be capable of robust growth within a human host, and hence

be close to highly dangerous forms. Natural-chirality microbes that exhibit reduced immune reactions could represent alternative chassis for live-cell therapeutic applications that pose much lower biosafety and biosecurity concerns.

(Nielsen & Keasling, 2016; Ro *et al*., 2006), the detection of specific chemical compounds (Chiang & Hasty, 2023; Riglar & Silver, 2018), and the *in situ* production of therapeutics to ameliorate disease (Riglar & Silver, 2018).

Some bacteria are most readily engineered by introducing plasmids—extrachromosomal circular DNA that can replicate independently from the bacterial genome—while others readily integrate introduced DNA sequences into their genomes. Researchers can rapidly generate suitable genes through chemical DNA synthesis and *in vitro* assembly methods, which can then be transformed into bacterial hosts within a few days. The process is routinely practiced by biology undergraduates and, in some cases, high school students (Gronvall, 2016; Jackson *et al*., 2019). It can also be done with minimal laboratory equipment by citizen scientists and biohackers (Gronvall, 2016).

The synthesis of mirror genes and the transformation of mirror bacteria could be relatively straightforward for anyone with access to synthetic mirror DNA and—for larger constructs requiring assembly—a small set of mirror enzymes. Mirror DNA is already commercially available, although it is more expensive than normal DNA. While mirror enzymes are currently only available in a small handful of laboratories, they would likely be much more accessible by the time the first mirror cells are created. Alternatively, advances in protein engineering could allow natural-chirality enzymes to perform the required functions in the future. Transformation methods such as electroporation or heat shock are not sensitive to chirality and could be used to introduce synthetic mirror DNA into a mirror bacterium in a straightforward manner.

Some applications in synthetic biology require more extensive engineering of the bacterial genome. In model organisms such as *Escherichia coli,* numerous methods permitting the rapid and facile editing of bacterial genomes have been developed using tools such as recombineering (Sharan *et al*., 2009; Wang *et al*., 2009) or CRISPR-Cas9 (Teng *et al*., 2024). In extreme cases involving thousands of changes, it can be more economical to synthesize the entire genome from scratch, as discussed in the following section. Many of these techniques require only the introduction of specific plasmids or synthetic DNA and hence could be adapted to mirror bacteria in a straightforward manner with access to synthetic mirror DNA. However, more complex manipulations may require skilled practitioners, greater quantities of synthetic DNA, or additional enzymes.

Finally, bacterial fitness can be improved and bacteria can be adapted to desired environments by adaptive laboratory evolution, which would work equally well for natural-chirality or mirror bacteria. In this process, scientists continuously passage a bacterial strain under selective conditions of their choice, such as a specific growth medium containing unusual nutrients or abiotic stresses (Sandberg *et al*., 2019). This simple process can yield substantial increases in fitness for strains that are

otherwise fragile and grow slowly due to large-scale genome modifications such as recoding or genome reduction (Hemez *et al*., 2024; Moger-Reischer *et al*., 2023).

Adaptive evolution can also optimize novel metabolic pathways, conferring the ability to utilize otherwise inaccessible nutrients. For example, *E. coli* strains have been evolved to grow on suboptimal or even non-native carbon sources (Fong *et al*., 2003; Herring *et al*., 2006; Lee & Palsson, 2010). Evolution also occurs in the absence of intentional human effort: as described in Section 8.4, evolution within the natural environment could exacerbate the risks from mirror bacteria if they escape confinement.

Any mirror bacterium would facilitate the creation of diverse mirror bacterial strains

The first mirror bacterium, if created, could be similar to a mirror *Mycoplasma* or *E. coli*, perhaps with a reduced or minimal genome or other genetic changes to facilitate the booting process, as discussed in Chapter 2. Because strains with highly modified or reduced genomes can be fragile and difficult to study, a logical next step would involve restoring missing genes and removing any artifacts of the boot process. A minimal *E. coli*, for instance, could be converted into mirror analogs of more robust and useful strains by introducing genes that mirror those found in other *E. coli*. For instance, researchers might wish to create mirror versions of *E. coli* K-12, BL21, and Nissle 1917, strains that are widely used for science, bioproduction, and medicine.

Creating a diverse range of mirror bacterial species would be a logical further step: mirror *Bacillus subtilis* or mirror cyanobacterium *Synechococcus elongatus* would offer different properties and enable different applications compared to mirror *Mycoplasma* or *E. coli*. While it is plausible that such mirror bacteria could be created from scratch using analogous techniques to that of the original mirror bacterium, it is also possible that such mirror bacteria could be created by converting existing mirror bacteria.

In 2010, researchers demonstrated that it is possible to convert *Mycoplasma capricolum* into the closely related *Mycoplasma mycoides* by transplanting a chemically synthesized *M. mycoides* genome into a *M. capricolum* recipient cell (Gibson *et al*., 2010). Genomic transplants between *M. capricolum* and more distantly related members of the *Spiroplasma* genus have also been performed, though the likelihood of success appears to be inversely related to phylogenetic distance (Labroussaa *et al*., 2016). Large-scale hybrids of *E. coli* and *Salmonella enterica* have also been created, in some cases without any significant reduction in growth fitness (Bartke *et al*., 2021), ultimately replacing over 90% of the genome and effectively transforming the initial bacterium into a species from a different genus without the need for genomic transplantation. Conversion between more distant species remains poorly explored, but the creation of a *Bacillus subtilis* bacterium containing the entire genome of the cyanobacterium *Synechocystis* PCC6803 suggests a potential path via switching the 16S ribosome to the new strain (Itaya *et al*., 2005). Ongoing advances in DNA synthesis (Hughes & Ellington, 2017) and synthetic genomics (Schindler *et al*., 2018) will likely make the process easier in the coming decades. While much remains unknown about the versatility and robustness of interspecies conversion methods, the successful creation of the first mirror bacterial species could enable the creation of additional mirror bacterial species even absent independent “boots”.

Fitness-enhancing capabilities can be ported from existing organisms

Natural organisms contain a diverse array of genes and functional capabilities that bioengineers could draw upon to create novel and diverse mirror bacterial strains. A natural-chirality gene can be mirrored by creating mirror DNA with an identical genetic sequence. By chiral symmetry, mirror genes in a mirror organism would function as mirror images of their natural-chirality counterparts in a natural-chirality organism. For example, genes allowing the catabolism of D-lactose or D-mannitol would, if encoded into mirror DNA, allow a mirror bacterium to catabolize L-lactose or L-mannitol.

As a result, creating metabolic pathways for the catabolism of natural-chirality molecules within mirror bacteria is not straightforward. Conferring the ability to catabolize natural-chirality D-glucose would require a metabolic pathway that would, in natural-chirality bacteria, allow the catabolism of mirror-image L-glucose. This ability is not found in common lab strains (including *E. coli*). It is, however, possible to isolate bacteria with this capacity from soil samples (Shimizu *et al*., 2012; Yachida & Nakamura, 2024), and the genes comprising one such utilization pathway in *Paracoccus laeviglucosivorans* have been characterized *in vitro*. By encoding genes for this pathway into mirror DNA along with a suitable transporter from an L-glucose catabolizer, D-glucose catabolism could be engineered into mirror bacteria, then evolved or engineered for higher metabolic efficiency. Given that D-glucose is far cheaper and more abundant than L-glucose, engineering D-glucose catabolizing strains would be a logical aim for nearly any potential application—but this modification would greatly increase the anticipated fitness of the mirror bacterium in the environment by enabling it to utilize the most abundant natural sugar.

Pathways exist to catabolize the mirror versions of other common sugars, including D-arabinose (LeBlanc & Mortlock, 1971), L-fructose (Park *et al*., 2007), L-galactose (Zhu & Lin, 1987), L-glyceraldehyde (Zhu & Lin, 1987), L-ribose (Trimbur & Mortlock, 1991), and L-xylose (Usvalampi *et al*., 2012; see also Table 1.3). The ability to catabolize mirror-image D-amino acids is also common in natural bacteria (see Table 1.2 for D-amino acid catabolism in *E. coli*), often proceeding through amino acid racemases that interconvert L- and D-amino acids (Miyamoto & Homma, 2021). Such racemases could similarly allow mirror bacteria to catabolize the ubiquitous canonical L-amino acids.

Abilities that require chiral interactions between mirror macromolecules and natural macromolecules would be more challenging to engineer. For instance, mirror proteases will not digest normal-chirality proteins (as discussed in Chapter 1), mirror antibodies will not bind to the same ligands as their natural-chirality counterparts, and a mirror AB toxin would be unlikely to function against natural cells. The *de novo* design of D-peptides to bind to specific natural-chirality peptide and protein targets has recently been demonstrated (Sun *et al*., 2024). As protein design tools improve, it should be possible to create mirror proteins with more complex activities and specificities, though such mirror proteins would not necessarily share high sequence and structure similarity with any existing natural proteins.

3.2 Biocontainment approaches might reduce accident risk, but they would face challenges

Given that (1) mirror bacteria might become synthetically accessible within the next few decades, (2) once created, engineering more robust and diverse mirror bacteria would be comparatively straightforward, and (3) a sufficiently robust and metabolically flexible mirror bacterium released into the environment could have severe consequences, mirror bacteria pose serious potential biosafety and biosecurity challenges. Biocontainment techniques might reduce accident risk from mirror bacteria, but securing them against misuse appears more challenging.

Physical containment would likely be insufficiently robust to prevent leaks of dangerous mirror bacteria

Biologists have long worked with dangerous pathogens. Such work can provide substantial benefits, enabling the behavior of pathogens to be better understood, and in some cases, the development of vaccines and other countermeasures. However, such work can pose risks to both the scientists performing the research and to others in the community who could be inadvertently exposed to the pathogen. Over time, biosafety practices have developed in order to best mitigate risks while still allowing vital research to be conducted.

When work on dangerous pathogens is required, strict physical containment measures within special biosafety laboratories are used to minimize risks. Nevertheless, even highly safe laboratories (e.g., with the BSL-4 designation) are susceptible to accident risk, and a number of such cases have been reported (Byers & Harding, 2016; Gillum *et al*., 2016; Manheim & Lewis, 2022). Therefore, physical containment alone is typically considered insufficient to permit experiments with biological agents that could have catastrophic consequences. For example, the scientific community has abstained from work involving the recreation or modification of smallpox (World Health Organization, 1980, 1996, 2010), which was eradicated in 1977.

Most of the highest-risk biological research is conducted on obligate pathogens, where protecting lab workers from infection is the highest priority. While BSL-3 and 4 laboratories do include measures to sterilize waste material and prevent the escape of aerosols, it is difficult to know how often such measures fail because most accidental releases do not result in detectable consequences. There are some well-known exceptions in which accidental leaks did result in visible infections. For instance, the last known death from smallpox, that of Janet Parker in 1978, was almost certainly due to a laboratory leak (UK Department of Health and Social Security, 1980). The 2007 UK outbreak of foot and mouth disease is also likely to have originated from a lab, and probably entered the environment through leaky pipes (Spratt, 2007).

A sufficiently robust mirror bacterium, unlike a virus, could potentially replicate independently within the environment, even absent a suitable host (see Chapter 8). Therefore, any failure of ventilation or waste sterilization systems could potentially be catastrophic. Given the rate of known laboratory-acquired infections, which likely reflects a higher rate of unknown accidental leaks of

biological material with or without infection, current physical containment measures do not appear sufficiently robust to permit work with potentially dangerous mirror bacteria.

Biological containment approaches might reduce the likelihood of environmental escape but would not alone be sufficient to achieve effective biosafety or biosecurity

Synthetic biologists have long been concerned with the escape of bioengineered natural-chirality microbes and have begun developing techniques to contain such organisms. Unlike physical containment, biological containment involves modifying the organism itself so that it is incapable of replicating in the external environment without the addition of specific chemical compounds. Such measures could allow the development of mirror bacteria with a reduced likelihood of environmental escape, providing greater robustness compared to physical containment (though implementing physical containment measures would still provide an additional layer of protection).

A common technique for the biocontainment of engineered bacteria is to make their growth and survival dependent on the supply of a specific metabolite—a metabolic auxotrophy. For example, genes for the biosynthesis of key metabolites like nucleosides and amino acids can be disrupted, rendering the bacterium dependent on the supply of a missing metabolite that it cannot produce itself (Bahey-El-Din *et al*., 2010; Ronchel & Ramos, 2001; Steidler *et al*., 2003). This system is straightforward to implement, but suffers from several drawbacks in natural-chirality bacteria: the auxotroph might find the missing nutrients in some natural environments or regain the ability to produce them by horizontal gene transfer. In a mirror bacterium, these drawbacks can be largely eliminated, but care would be required to create systems that are robust to human error.

Many mirror molecules are thought to be absent from the natural environment, which should make it possible to create auxotrophs that definitely could not survive in nature. Careful experimentation with natural-chirality bacteria could be used to verify that these auxotrophs could not escape using unexpected metabolic pathways. For instance, though D-amino acids are not as common as L-amino acids, they do occur naturally—and a subset is known to fulfill specific biological functions (Genchi, 2017). Furthermore, as discussed in Chapter 1, many bacteria can utilize both amino acid enantiomers due to promiscuous transporters and enzymes (see Table 1.2). With careful design, it should be possible to identify metabolic auxotrophies that would avoid these difficulties and minimally disrupt cell functioning. L-pantothenate, for instance, might be absent in nature. Since some natural-chirality bacteria already lack D-pantothenate biosynthesis and instead obtain D-pantothenate from the environment (Gerdes *et al*., 2002), the pathway is dispensable. Lipoic acid and biotin auxotrophy are other plausible options. Multiple distinct auxotrophies could be used simultaneously to increase robustness.

Though mirror bacteria cannot obtain genetic material from natural-chirality organisms, gene transfer between mirror bacterial strains is possible. In particular, if mirror bacteria with different metabolic auxotrophies were created, they may inadvertently come into contact and transfer genetic material, creating a mirror bacterium that is no longer auxotrophic. Technical interventions to prevent horizontal gene transfer could help to mitigate this risk. Additionally, human error during attempts to

switch an auxotrophy or introduce additional biosynthetic capabilities could also lead to accidental escape.

While metabolic auxotrophy is a straightforward way to create a mirror bacterium with reduced escape risk, other auxotrophic biocontainment methods have been developed. One approach involves re-engineering multiple essential cellular proteins to require the incorporation of an unnatural non-canonical amino acid in order to function, creating a form of 'synthetic auxotrophy' (Mandell *et al*., 2015; Rovner *et al*., 2015). If properly implemented, escape from synthetic auxotrophy through horizontal gene transfer or other evolutionary mechanisms would be very unlikely. Such biocontainment would, however, be more technically challenging to implement. While non-auxotrophic containment methods exist, they are unlikely to be suitable for mirror bacteria because they are vulnerable to the gradual acquisition of individual mutations, even taking into account efforts to stack multiple non-auxotrophic methods or implement interventions to reduce the rate of evolutionary drift (Brophy & Voigt, 2014; Calles *et al*., 2019; Gallagher *et al*., 2015). Future advances could yield superior biocontainment strategies.

Biological approaches to biocontainment have been developed for microbes used in industrial or medical applications. In these cases, environmental escape is undesirable, but generally not expected to cause major consequences for humans or ecosystems. In contrast, it appears that the accidental release of mirror bacteria could have potentially severe consequences for humans and the environment (see subsequent chapters). While effective biocontainment using the approaches discussed above might reduce the escape risk by many orders of magnitude, a low accident risk due to human error or unexpected evolutionary events would persist even if highly advanced biocontainment approaches were implemented. If mirror bacteria prove highly dangerous, this residual risk may still outweigh the benefits of their scientific and technological applications.

An effective biosafety regime would require not only methods to robustly biocontain mirror bacteria, but also strict provisions to prevent these biocontainment measures from being disabled by accident or recklessness. Whether governance mechanisms exist that could lower the risk to an acceptable level, and whether there are realistic implementations of such measures that could be effectively implemented across the many jurisdictions where work on mirror bacteria might occur, lies beyond the scope of this report. We will, however, briefly discuss the biosecurity challenges raised by mirror bacteria, which appear particularly severe.

3.3 Creating robustly biosecure mirror bacteria is not feasible

Terrorist groups, including Al-Qaeda (Parachini & Gunaratna, 2022), the Islamic State (Parachini & Gunaratna, 2022), and Aum Shinrikyo (Danzig *et al*, 2011), are known to have pursued biological weapons. Offensive biological weapon development is banned by the 1972 Biological Weapons Convention (United Nations, 1972), but several states have in the past nevertheless clandestinely pursued such weapons illegally, including Iraq under Saddam Hussein (United Nations Monitoring, Verification and Inspection Commission, 2007), apartheid South Africa (Gould & Folb, 2002), and the Soviet Union (Leitenberg & Zilinskas, 2012). The possibility that similar actors might pursue mirror bacteria as potential weapons is deeply concerning. Though any danger posed by mirror

bacteria would likely cause indiscriminate harm to humans and natural ecosystems, they might still be pursued for blackmail by rogue governments or by non-state groups that see such mass harm as intrinsically desirable. Given that the misuse of mirror bacteria could result in especially grave consequences for humans and many ecosystems, mirror biogenesis would require robust biosecurity protection. Unfortunately, devising a form of biocontainment that could resist deliberate jailbreaking attempts by skilled individuals with access to mirror DNA appears exceptionally difficult.

Any bacterial auxotrophy resulting from missing or conditionally functional genes can be removed by adding easily identified functional versions of those genes through bacterial transformation. Therefore, a person with moderate molecular biology training could release bacteria from most forms of auxotrophy. Given access to mirror DNA—which could be generated from commercially available mirror phosphoramidites using a standard nucleic acid synthesizer—they could achieve the same outcome for a mirror bacterium. Depending on the mirror bacterial species, the type of biocontainment implemented, and the skill of the practitioner, it might take weeks or even months to remove multiple auxotrophies, especially if many genome modifications are required. Still, since the skill and resource requirements are limited, many actors could remove biocontainment measures with relative ease. More elaborate biocontainment strategies, such as synthetic auxotrophy for an unnatural amino acid in a recoded mirror bacterium would be more complex to reverse, requiring the replacement of every essential gene rendered dependent upon a non-canonical amino acid, but could still be disabled by a sufficiently resourced actor. Alternatively, if a known biosynthetic pathway for the non-canonical amino acid is available or newly discovered, it could be engineered into the mirror bacterium (Butler *et al*., 2023).

Even if robust biocontainment strategies could make jailbreaking mirror bacteria difficult, several important asymmetries between attack and defense make achieving security through such measures challenging. Defenders must block every possible pathway by which extant mirror bacteria could be weaponized, while malicious actors simply need a single viable path to weaponization. A single mistake could create an unpatchable hole. Moreover, defenders need to create mirror cells that remain safe indefinitely, regardless of how other biotechnologies develop. Feats that seem impossible now might become routine in coming years and decades, so mirror bacteria that appear robustly biosecure today may not remain so forever—especially given the potential for malicious actors to directly generate weaponized mirror bacteria by following established protocols for mirror biogenesis.

Given that even fragile or rigorously biocontained mirror bacteria could be weaponized by malicious actors, preventing their access to mirror bacteria would be imperative. Existing access controls for dangerous pathogens are imperfect, and given the potential for far more severe consequences from an intentional mirror bacterial release, any access controls on mirror bacteria would need to be very restrictive. Further analysis of potential access control measures and whether they could be feasibly implemented across jurisdictions where mirror bacterial research might occur, is beyond the scope of this technical report.

Chapter 4: Risks to Human Health

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The previous chapters described the potential pathways by which mirror bacteria could be created and modified. The remainder of the report focuses on the potential risks that such mirror bacteria could pose to humans, animals, plants, and the environment. This chapter focuses on risks to human health, while the next chapter discusses potential medical countermeasures that could protect humans from mirror bacteria.

Humans have evolved complex immune mechanisms to combat bacterial pathogens; many, though not all, of these mechanisms appear unlikely to function properly against mirror bacteria. If a generalist mirror bacterium as described in Chapters 2 and 3 entered the human body, the initial infection would likely be extracellular because it would lack the adaptations typically needed to survive and proliferate as an intracellular bacterial pathogen. For this reason, we focus on immune mechanisms relevant to extracellular bacteria throughout this chapter.

Section 4.1 describes pathogen recognition, which generally relies on stereospecific interactions between immune receptors and specific microbial macromolecules. The macromolecules within mirror bacteria would likely be unable to properly trigger these receptors, and this could greatly impair the immune response.

Section 4.2 describes the innate immune effectors that target pathogens. Mirror bacteria would likely be partially or fully resistant to many of these effectors, including antimicrobial enzymes, professional phagocytes, and the complement system.

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Section 4.3 describes the adaptive immune response, which could also be significantly weakened. In particular, it is plausible that deficiencies in dendritic cell activation and antigen presentation would result in a failure to activate T cells, which would inhibit both cellular and humoral (antibody) immune responses.

As discussed throughout Sections 4.1–4.3, humans with even partial immunodeficiencies are often at greatly heightened risk of serious disease or death from bacterial infection, including from bacteria that are not usually pathogenic for healthy adults. Similarly, natural specialized pathogens commonly exhibit high degrees of immune evasion in order to establish infection. The unique biology of mirror bacteria could allow them to evade immune mechanisms to a possibly unparalleled extent. Natural pathogens typically utilize virulence factors—bacterial proteins that interact with host ligands to facilitate bacterial adhesion or invasion, or that can directly induce damage. Mirror bacteria would not, absent deliberate engineering, possess virulence factors, and most virulence factors copied from natural-chirality pathogens would not function due to chirality incompatibilities with host ligands. But even in the absence of functional pathogenic virulence factors, their capacity for immune evasion raises the possibility that a mirror *Escherichia coli* or similar mirror generalist bacteria could act as a potentially lethal human pathogen.

Section 4.4 describes the mechanisms by which mirror bacteria could enter the human body without the need for functional virulence factors. Human barrier defenses are imperfect and commensal bacteria can be passively translocated across physical barriers into the bloodstream in healthy individuals. Similar translocations would likely occur with sufficient exposure to mirror bacteria, although it is very uncertain how many mirror bacteria would be needed to establish an infection. Mirror bacteria would lack specialized adhesion mechanisms or other specialized mechanisms that facilitate invasion. This could impair but not necessarily preclude colonization of human barrier surfaces like the lung, and adhesion does not appear necessary for infection or translocation into other bodily regions.

Section 4.5 describes the potential consequences of mirror bacteria within the bloodstream. Given that nutrients and abiotic conditions within blood are plausibly growth-permissive to a generalist mirror bacterium and that the normal immunological clearance mechanisms are likely significantly impaired, it is plausible that mirror bacteria within the blood could grow exponentially. Both the reduced immunological recognition and absence of virulence factors could result in a disease progression that is difficult to predict but likely highly unusual. Nevertheless, it is plausible that at high mirror bacterial concentrations, residual immunological recognition would lead to a lethal sepsis-like hyperinflammatory response. Even if this is avoided, other consequences of infection could potentially be fatal or otherwise highly detrimental to human health.

In summary, sufficient exposure to mirror bacteria may be a life-threatening event, even if the mirror bacteria have no particular adaptations or virulence factors for human infection (Figure 4.1). Deliberate misuse could increase this danger further.

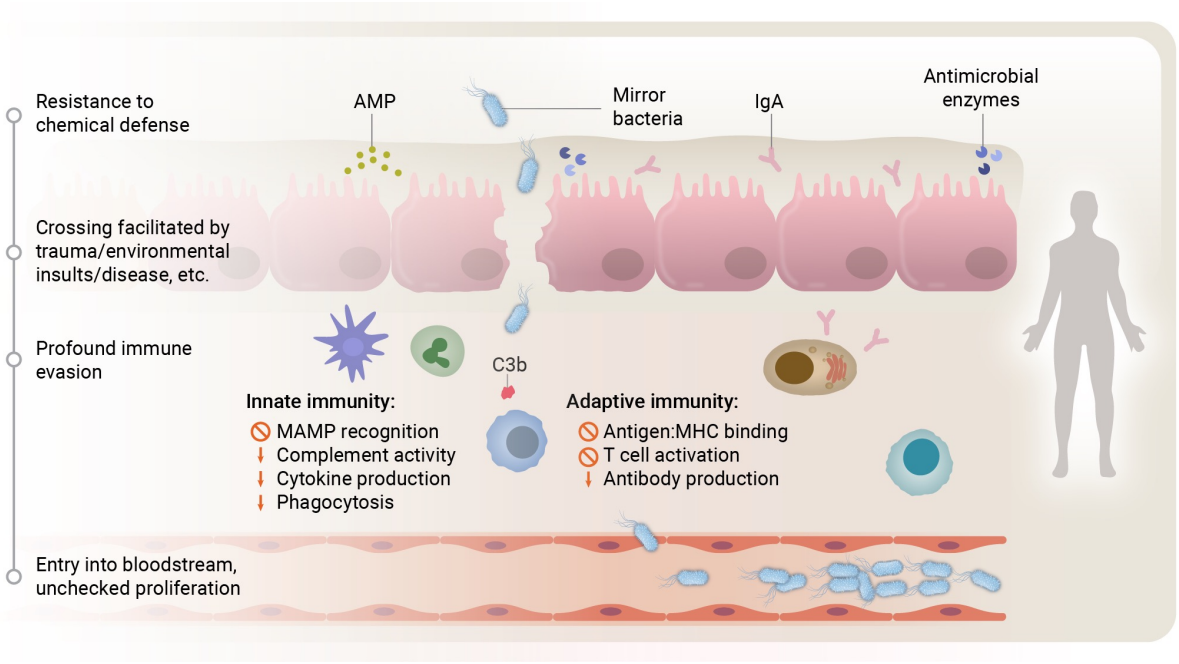

Figure 4.1: The human health risks of mirror bacteria
Mirror bacteria that encounter humans may be able to survive and potentially colonize boundary surfaces like the skin and gut, aided by their reversed chirality granting resistance to defenses such as secreted antimicrobial peptides (AMPs), antimicrobial enzymes, and immunoglobulin A (IgA). Mirror bacteria at these surfaces may translocate into the body through transient breaches in physical barriers, which occur frequently from a variety of causes. The reversed chirality of mirror bacteria may prevent the innate immune system from detecting mirror microbe-associated molecular patterns (MAMPs) and the adaptive immune system from processing and presenting mirror peptide antigens via major histocompatibility complex (MHC) molecules. Downstream defenses, such as complement, phagocytosis, and antibody production would also be impaired. Since mirror bacteria may not be effectively cleared by the immune system, this could allow entry into the bloodstream and unchecked proliferation therein.

4.1 Innate immune detection of mirror bacteria could be significantly impaired

Most pattern recognition receptors could have severely impaired ability to recognize mirror versions of their ligands

Pathogen recognition is the first step required for an effective immune response. Bacterial pathogens are typically first detected by macrophages and dendritic cells present in submucosal tissues, such as alveolar macrophages in the lung or Langerhans and interstitial dendritic cells in the skin[4]. These immune cells express pattern recognition receptors (PRRs) that bind to microbe-associated molecular

[4] There are many other immune cell subsets within major lineages, often resident in specific tissues, that are too numerous to cover in this report. Therefore,we have limited discussion to responses across major cellular lineages, with the exception of certain cell subsets that appear particularly important for understanding immunological responses to mirror bacteria.

patterns (MAMPs). MAMPs are molecules that are highly conserved across a broad range of microbes but otherwise absent in animals, making them reliable indicators of potential pathogens[5]. The binding of MAMPs by PRRs is a critical step in the activation of both the innate and the adaptive immune systems. PRR engagement activates macrophages, causing them to become highly phagocytic and secrete cytokines that induce inflammation, leading to an influx of neutrophils to the infection site. Dendritic cells are also activated when their PRRs engage MAMPs. Activation causes these professional phagocytes to increase antigen uptake and migrate to lymphoid tissues, where they present antigens to T cells to initiate adaptive immunity.

Almost all known bacterial MAMPs detected by human PRRs are chiral. Standard PRRs such as Toll-like receptors (TLRs), for example, bind to chiral molecules such as lipopolysaccharide (LPS), proteins, peptidoglycan, various lipoproteins, and CpG DNA (Kawai & Akira, 2010). Other key PRRs, including members of the nucleotide-binding domain and leucine-rich repeat containing (NLR) family, bind to flagellin, proteins from bacterial secretion systems, and peptidoglycan degradation products (Muñoz-Wolf & Lavelle, 2016), which are also chiral. Formyl peptide receptors, which recognize chiral f-met peptides from bacteria[6], enable phagocytes—especially neutrophils—to home to the site of infection (He & Ye, 2017). In addition, chiral molecules play a dominant role in non-canonical immunosurveillance mechanisms. For example, the semi-invariant T cell receptor (TCR) in mucosal-associated invariant T cells recognizes transient chiral intermediates of bacterial riboflavin metabolism (Corbett *et al*., 2014). Because protein interactions are stereospecific (see Chapter 1), we would expect binding between many chiral PRRs and the mirror-images of these MAMPs to be significantly impaired (Figure 4.2).

There might be some PRRs that could plausibly bind mirror MAMPs, particularly at high MAMP concentration, or for repetitive ligands where avidity effects could amplify weak residual interactions. For instance, high concentrations of mirror dsRNA appear to activate the PRRs TLR3 and TLR7 (Yu & Sczepanski, 2023), though dsRNA is typically a viral MAMP and so its potential relevance for a mirror bacterial infection is unclear. In addition, scavenger receptors on macrophages can bind to many different kinds of substances, including abiotic ones (Alquraini & El Khoury, 2020), and may be capable of recognizing certain mirror macromolecules.

Lipid antigens may potentially be less sensitive to changes in chirality than most of the MAMPs discussed above. For example, TLR2 is unusually promiscuous in its binding of diacylated and triacylated lipopeptides found in bacterial membranes. Most contacts between TLR2 and its ligands form with the achiral acyl chains of the lipopeptides, though some bonds are dependent on the chiral glycerol backbone carbon and the chiral amino acids in the peptide chain (M. S. Jin *et al*., 2007). Lipopeptides with reversed chirality at some of these carbons are able to activate TLR2, albeit with greatly reduced potency. It remains unclear if fully mirrored lipopeptides could activate TLR2

[5] The molecules sensed by PRRs are often referred to as “pathogen associated molecular patterns” or PAMPs (Janeway, 1989). In this report, we use MAMP to acknowledge that these molecules are found in pathogenic and non-pathogenic microbes alike.

[6] Bacterial proteins start with formyl methionine (f-met), but eukaryotic proteins do not. This makes f-met an effective MAMP.

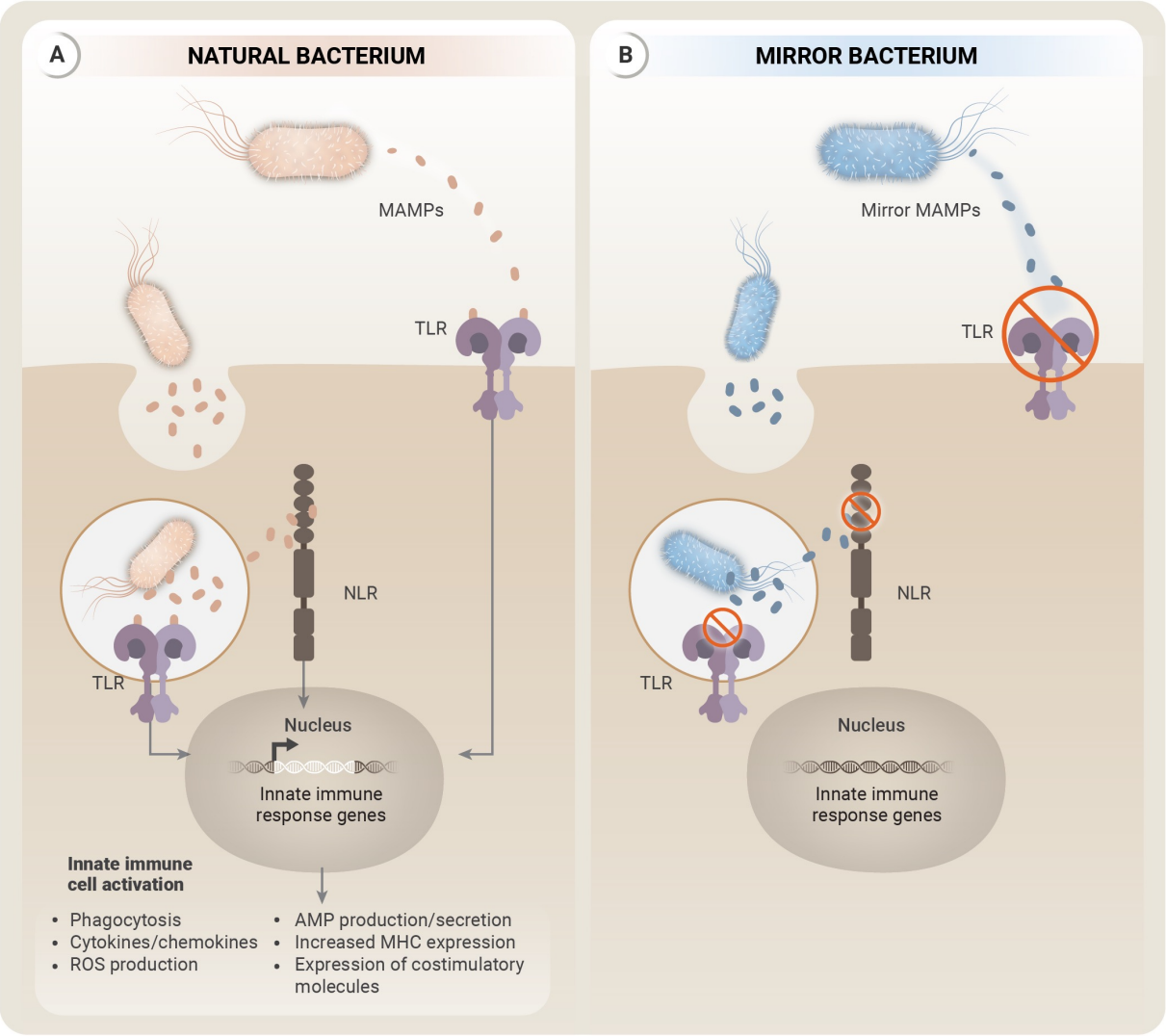

Figure 4.2: Innate immune recognition of mirror bacteria could be severely impaired
A. Natural-chirality bacteria produce MAMPs, which are conserved molecules that are recognized by various PRRs, including TLRs and NLRs. These receptors are present on the cell surface, in endosomes, and in the cytosol. PRR activation triggers the upregulation of innate immune response genes, which results in increased phagocytosis by immune cells, increased cytokine and chemokine expression, and many other responses. **B.** Mirror bacteria would produce mirrored versions of these MAMPs. PRR-mediated detection of mirror MAMPs is expected to be severely impaired because almost all known bacterial MAMPs detected by human PRRs are chiral. Impaired PRR activation would likely result in reduced or failed induction of innate immune response genes.

signaling (Metzger *et al*., 1991; Takeuchi *et al*., 2000). Similarly, LPS is detected by TLR4 and MD2 via interactions with the acyl chains and phosphate groups of the lipid A component. Lipid A is chiral, but its long, flexible acyl chains that mediate a majority of the contacts with MD2 and TLR4 are achiral (B. S. Park *et al*., 2009). Experimental data with enantiomeric LPS is lacking, so it is ultimately unclear how sensitive LPS binding to TLR4/MD2 would be to changes in chirality. Thus, while most PRRs would likely have severely impaired ability to recognize mirror MAMPs, uncertainty remains around how much residual PRR activation could be triggered by a mirror

bacterial infection, particularly at high concentrations of MAMPs. Empirical evidence from laboratory experiments using mirror MAMPs or computational modeling could help address these uncertainties.

While critical PRR pathways would likely be compromised by mirror biology, we are aware of at least one achiral MAMP: the bacterial molecule (E)-4-Hydroxy-3-methyl-but-2-enyl pyrophosphate (HMBPP). HMBPP is typically produced as intermediate in bacterial metabolism (Boucher & Doolittle, 2000), and some bacteria use an alternative metabolic pathway which does not produce HMBPP (Heuston *et al*., 2012; C. Wang *et al*., 2017; R. Yoshida *et al*., 2020). When released by intracellular bacteria, HMBPP binds to the cytoplasmic domain of a membrane protein called butyrophilin, inducing a conformational change that allows it to dimerize with a second butyrophilin protein and be detected by Vγ9/Vδ2 T cells, a class of cells which bridge innate and adaptive immunity (Uldrich *et al*., 2020). It is possible that other achiral MAMPs may be discovered as the human PRR repertoire is further characterized. It seems unlikely, however, that HMBPP recognition or other, unknown, achiral MAMPs would themselves suffice to produce an effective innate immune response.

Alongside MAMPs, elements of the innate immune system can be triggered when PRRs and other receptors detect damage-associated molecular patterns (DAMPs) generated by host-cell damage and tissue disruption (Gong *et al*., 2020). As DAMPs arise from the natural-chirality host rather than from pathogen particles, DAMP detection would function normally. Given mirror bacteria would generally lack virulence factors that could directly damage host tissue, it seems unlikely that significant host-cell damage would arise in the early stages of a mirror bacterial infection unless damage-causing virulence factors were intentionally engineered into the bacterium. At the relatively late stages of infection where tissue damage would probably occur, it appears unlikely that DAMP-mediated inflammation alone would impede bacterial proliferation. DAMPs are not sufficient to combat bacterial infections in PRR-deficient animal models or patients, as described below. As such, DAMP-induced inflammation may instead exacerbate harms to the patient (see Section 4.5).

Compromised immune detection of mirror bacteria could result in vulnerability to infection

The expected inability of much of the human PRR repertoire to respond to mirror ligands suggests that the innate immune system's ability to detect mirror bacteria would likely be markedly impaired. Evidence from experiments, analogous human immunodeficiencies, and the clinical consequences of PRR masking observed in natural pathogens suggests this loss would result in substantial vulnerability to mirror bacterial infection.

Various natural-chirality bacterial pathogens have evolved mechanisms to shield surface MAMPs from detection by their corresponding PRRs. For example, certain *Helicobacter pylori* strains express tri- or tetra-acylated LPS instead of hexa-acylated LPS, which are less immunostimulatory (Chmiela *et al*., 2014; Tran *et al*., 2005). *Salmonella* and *Yersinia pestis* are both able to alter their LPS composition in order to make them less stimulatory during infection (Kawasaki *et al*., 2004; Telepnev *et al*., 2009). Various *Proteobacteria*, including some strains of *Campylobacter jejuni* and *H. pylori*,

express flagellin that is significantly less able to stimulate TLR5 signaling than flagellin from other bacterial species (Andersen-Nissen *et al*., 2005). Many commensal bacteria also express flagellins that weakly stimulate TRL5, which facilitates their persistence in the human gut (Clasen *et al*., 2023). These modifications, and others, have been found to enhance the ability of bacteria to evade the immune system and subsequently survive (Arpaia & Barton, 2013).

A range of mouse immunity studies show that mice with deletions in multiple PRRs are much more likely to die from a bacterial infection than healthy controls. For example, mice unable to express any TLRs succumb to bacterial infections that wild-type mice can clear (Sivick *et al*., 2014). Similarly, mice lacking the NLRs NOD1 and NOD2 (which recognize bacterial peptidoglycans) also die from *E. coli* infections that wild-type mice can survive (Y.-G. Kim *et al*., 2008). Finally, mice lacking formyl peptide receptors are more susceptible to bacterial infections than their wild-type counterparts (Gao *et al*., 1999; Liu *et al*., 2012).

Additionally, studies of mice with deficiencies in individual PRRs show the impact even one receptor failure can have on health. Mice lacking individual TLRs that recognize bacterial MAMPs carry increased bacterial burdens and are less likely to survive infection (Bhan *et al*., 2008; Feng *et al*., 2003; Sjölinder *et al*., 2008; Takeuchi *et al*., 2000; Torres *et al*., 2004). Similarly, mice lacking a single Nod-like receptor, NOD2, are much more vulnerable to bacterial infections than their wild-type counterparts (Deshmukh *et al*., 2009; Divangahi *et al*., 2008; K. Shimada *et al*., 2009).

The closest human analog to such a deficit in PRR function is the severe inherited defects across PRR signaling in those with IRAK-4 or MyD88 deficiency. Both IRAK-4 and MyD88 are downstream of and essential for most TLR signaling, and thus their deficiency disables much of the human PRR repertoire (Picard *et al*., 2003; von Bernuth *et al*., 2008). Individuals with either condition are extremely susceptible to pyogenic (pus-producing) bacteria, and often experience multiple life-threatening invasive infections during the first years of life (Picard *et al*., 2010). Roughly 40% of known cases die before reaching adulthood despite treatment for invasive infections and intensive use of vaccination, antibiotic prophylaxis, and immunoglobulin infusions (Picard *et al*., 2010). Survival dramatically improves from the second decade onwards, and both the frequency and severity of infections fade. This is hypothesized to be due to the maturation of other innate and adaptive immune mechanisms which can compensate for the lack of PRR signaling (Ku *et al*., 2007). However, as discussed throughout this chapter, these compensatory immune mechanisms are also unlikely to properly function against mirror bacteria.

IRAK-4 and MyD88 deficiencies do not perfectly mimic the mechanistic deficits that would be expected in response to mirror bacteria. For example, these deficiencies also impair immune signaling pathways other than TLR signaling, while leaving intact pathways that may be impaired against mirror bacteria (Picard *et al*., 2011). Nonetheless, evidence from both human immunodeficiencies and mouse models suggests that healthy individuals could be vulnerable to mirror bacterial challenge due to the limited ability of the innate immune system to detect mirror bacteria.

4.2 Mirror bacteria would likely be resistant to most innate immune responses

Detection of an infection by the immune system triggers a variety of responses that act to eliminate the invading microbes. In humans, these mechanisms include the secretion of antimicrobial proteins and peptides, the complement system, and phagocytosis. Activation of many of these mechanisms would likely be severely impaired because they are downstream of host receptors. Moreover, the molecular mechanisms of these responses, much like those for detection, often rely on stereospecific interactions between host and pathogen. Therefore, the reversed chirality of mirror bacteria would likely confer a high degree of resistance to these immune responses.

Some antimicrobial peptides could retain the ability to kill mirror bacteria, but their release would likely be impaired by failures of innate immune recognition

Antimicrobial peptides (AMPs) are a crucial component of the human innate immune system, serving as endogenous antibiotics that provide a first line of defense against invading pathogens. Humans express more than 100 antimicrobial peptides, many of which have not been characterized in great detail (G. Wang, 2014), and a detailed review of their functions is beyond the scope of this report.

Most AMPs can disrupt bacterial cell membranes via electrostatic and amphipathic interactions, which either destroy osmotic potential, create pores, or act in a detergent-like manner to break apart the phospholipid bilayer (Brogden, 2005). Such mechanisms are insensitive to chirality and should be able to target mirror bacterial membranes; this has previously been confirmed in experiments. For instance, the D-enantiomer of defensin HNP4 has similar antimicrobial properties to its natural counterpart (Wei *et al*., 2009), while the D-enantiomer of cathelicidin LL-37 has similar or somewhat reduced efficacy (Dean *et al*., 2011a, 2011b), suggesting both should retain potency against mirror bacteria.

Many AMPs, however, are pleiotropic in effect, acting via both generic membrane disruption and specific chiral targets. For instance, the human defensins HNP1 and hBD-3 both block the synthesis of peptidoglycan in the bacterial cell wall by binding to a chiral precursor called lipid II (de Leeuw *et al*., 2010; Sass *et al*., 2010). The D-enantiomer of HNP1 is significantly less bactericidal than natural HNP1 against *Staphylococcus aureus*, but not *E. coli* (Wei *et al*., 2009), which implies that HNP1 would retain efficacy against a mirror *E. coli* but not a mirror *S. aureus*. By testing D-enantiomers of other AMPs against common bacteria, it should be possible to ascertain which AMPs will retain efficacy against mirror bacteria.

However, given the likely defects in innate immune detection, it is plausible that AMPs would not be present in sufficient concentrations to be effective against mirror bacteria in most extracellular environments. Because many AMPs have toxic effects on mammalian membranes, their expression and secretion are generally tightly controlled and often rely on MAMPs, inflammatory cytokines, or other immunological signals (Gallo & Hooper, 2012). Impaired innate immune detection, described in Section 4.1, and defects in professional phagocyte and T cell activation, described below, could

therefore significantly reduce AMP concentrations in extracellular spaces. While some constitutive production and secretion does occur, particularly at mucosal surfaces (Laube *et al*., 2006; Muniz *et al*., 2012; Yarbrough *et al*., 2015), the fact that constitutively expressed AMPs do not appear to compensate for the loss of PRR signaling in humans with MyD88/IRAK4 or other immunodeficiencies suggests impaired innate immune recognition could substantially limit the overall impact of AMPs against mirror bacteria.

Antibacterial enzymes would likely have greatly reduced activity against mirrored substrates

Some antimicrobial proteins are enzymes that cleave bacterial molecules. Because enzyme catalysis is stereospecific, it is generally anticipated that many of these enzymes would have little activity against mirror molecules (Uppalapati *et al*., 2016). For example, experimental evidence indicates that D-peptides are partially or completely resistant to degradation by representatives from several of the major classes of proteases, including carboxypeptidase A, papain, pepsin, trypsin, elastase, and chymotrypsin (Guichard *et al*., 1994; Miller *et al*., 1995; Vaissière *et al*., 2017). Limited evidence also suggests that cathepsins are unable to efficiently cleave D-peptides (Chu *et al*., 2012; Meldal *et al*., 1998). This protease resistance has led to the development of D-peptides as a therapeutic drug class due to their increased half-life (Lander *et al*., 2023; Uppalapati *et al*., 2016).

The phospholipase A_2 (PLA_2) family is another class of antibacterial enzymes. These enzymes hydrolyze the sn-2 position of bacterial phospholipids present in cell walls, resulting in bacterial cell death (Murakami *et al*., 2016). Two secreted PLA_2 ($sPLA_2$) enzymes, PLA_2G2A and PLA_2G5, are upregulated and expressed in phagocytes and other cells after pathogen stimulation (Dabral & van den Bogaart, 2021). In addition to killing bacteria, the fatty acids and lysophospholipids they produce also trigger innate immunity in another positive feedback loop (Murakami *et al*., 2016). Given that $sPLA_2$ interacts with phospholipids in a chiral manner, mirror bacteria would likely be resistant to this mechanism: porcine and bee venom PLA_2 do not hydrolyze mirror-image phospholipids (Gudmand *et al*., 2010; Singh *et al*., 2010).

Lysozyme is an important antibacterial enzyme that cleaves peptidoglycan, the major structural component of the cell wall that protects bacteria from osmotic and environmental stressors (Callewaert & Michiels, 2010). Lysozyme is expressed at high concentrations in the mucosal epithelia, where it kills extracellular bacteria, and in the phagolysosomes of macrophages and neutrophils (Ragland & Criss, 2017). The peptidoglycan cleaved by lysozyme triggers innate immune responses through activation of extracellular and intracellular PRRs, initiating a positive feedback loop.

Lysozyme would likely fail to cleave mirror peptidoglycan. Although this has not been assessed experimentally, lysozyme's catalytic mechanism is well studied (Callewaert & Michiels, 2010; Ragland & Criss, 2017), and it seems unlikely that its active site could accommodate mirror peptidoglycan in a configuration that would permit hydrolysis. However, catalytically-disabled lysozyme still retains bactericidal activity through disrupting membranes (Derde *et al*., 2013; Ibrahim *et al*., 2001; Nash *et al*., 2006), similar to the action of many AMPs described previously. It is

therefore plausible that this bactericidal activity would function against mirror bacteria, though lysozyme has a strong affinity for lipopolysaccharide (Derde *et al*., 2015) and it is not clear whether lysozyme would be as effective at disrupting the outer membranes of a mirror Gram-negative bacteria. To our knowledge, mirror-image lysozyme—which could be used to definitively answer these questions—has not been chemically synthesized.

Knockout mice lacking lysozyme-M (one of two murine lysozyme proteins) have respiratory tracts with dramatically elevated levels of resident bacteria[7] and show defects in killing and eliminating bacterial infections (Cole *et al*., 2005; Davis *et al*., 2011; Ganz *et al*., 2003; Markart *et al*., 2004; Nash *et al*., 2006; J. Shimada *et al*., 2008). Many human commensals and pathogens have also evolved to evade lysozyme's catalytic activity, either by modifying peptidoglycan to reduce binding (Ragland & Criss, 2017; Vollmer & Tomasz, 2000) or by producing proteinaceous inhibitors (Clarke *et al*., 2010). This further underscores the importance of this catalytic activity as an immune defense.

Neutrophil granules also contain a number of other enzymes that act directly on bacterial components. These include serine proteases, cysteine proteases, neutrophil gelatinase-associated lipocalin, proteinase 3, cathepsin G, neutrophil elastase, CAP37, and NSP4 (Cowland & Borregaard, 2016). These would not be expected to properly function against mirror bacteria, which could markedly decrease the effectiveness of neutrophil-mediated bacterial killing.

Complement activity versus mirror bacteria could be partially impaired

The complement system is a set of blood proteins that, upon activation, produce proteins that can stimulate immune cells or attach to (opsonize) pathogens to aid their consumption by phagocytes. It can also directly harm Gram-negative bacteria by self-assembling into the membrane attack complex (MAC), which creates holes in the bacterial cell membrane, causing the cell to burst (Dunkelberger & Song, 2010). Complement system proteins are consistently present in the blood, and *in vitro* studies suggest complement can target, opsonize, and kill bacteria—either via the MAC or phagocytosis—in a matter of minutes to hours (Heesterbeek *et al*., 2018; Verbrugh *et al*., 1979; Zewde *et al*., 2016). Activation of the complement system occurs through three distinct pathways: the classical, alternative, and lectin pathways. In the context of mirror bacterial challenge, it appears the lectin pathway may be significantly impaired, the classical pathway could retain some activity through natural antibody binding, and the alternative pathway may remain largely intact (Figure 4.3).

The lectin pathway of complement activation is initiated when ficolins or collectins, such as mannose-binding lectin (MBL), recognize and bind to specific carbohydrate patterns on the surface of pathogens (Mastellos *et al*., 2024). This binding activates MBL-associated serine proteases (MASPs), which then activate the complement system. The specificity and affinity of lectin-carbohydrate bonds differs across lectins and can be very sensitive to the structure of the carbohydrate (Raposo *et al*., 2021). Therefore, it seems unlikely that human lectins would bind strongly to mirror carbohydrates. As a result, the lectin pathway would likely fail to activate.

[7] Markart *et al*. (2004) were able to culture 100–5 000 colony forming units of lactobacilli from lung homogenates of lysozyme-deficient mice, but nothing grew from lung homogenates of co-housed wildtype mice.

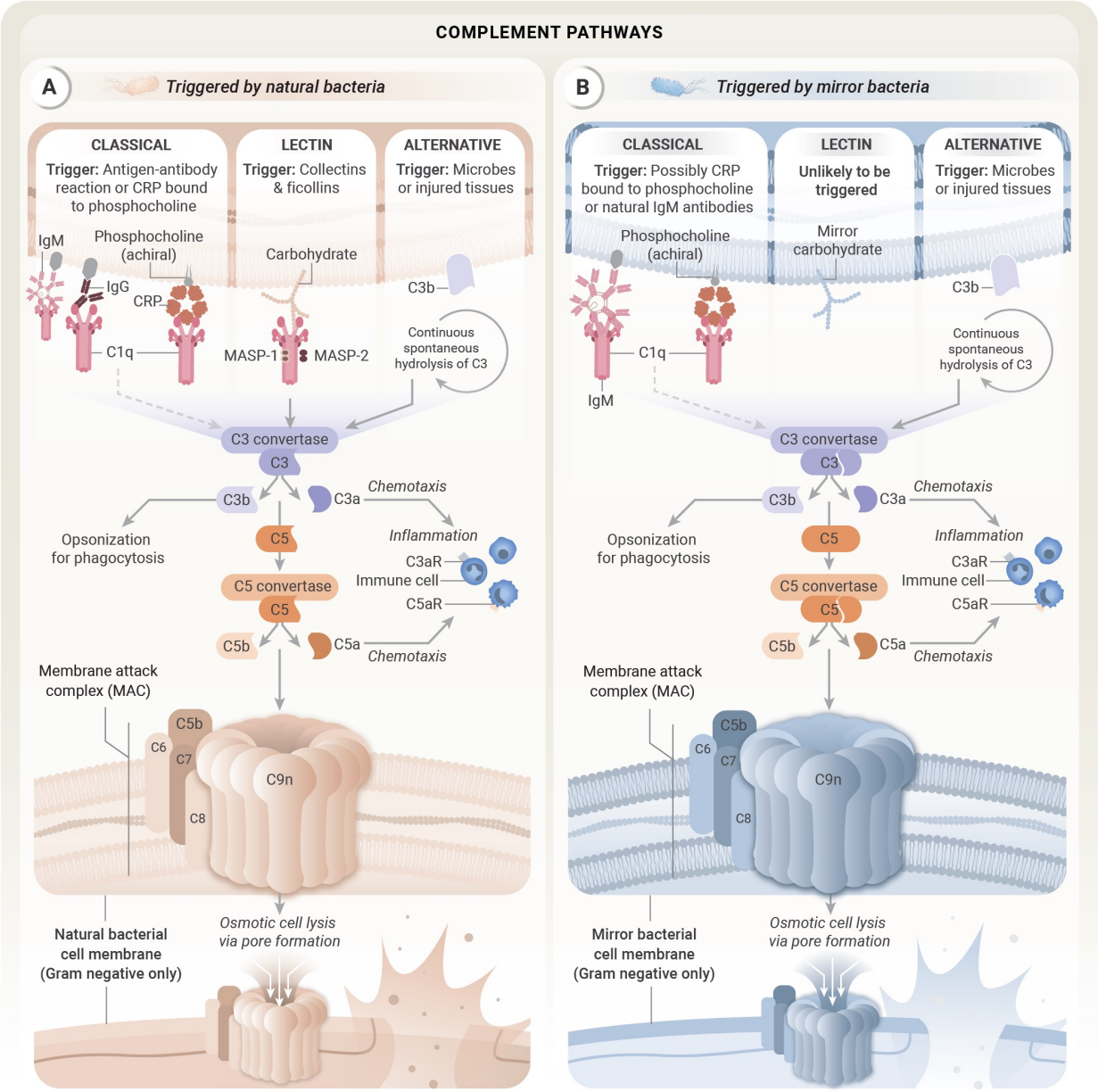

Figure 4.3: Complement activity against mirror bacteria would likely be impaired
A. Natural bacteria trigger three distinct pathways of complement activation. The classical pathway is triggered by circulating antibodies that bind to the surface of a bacterium and are then bound by the complement protein C1q. The lectin pathway is initiated when lectins, such as mannose-binding lectin (MBL), bind to specific carbohydrate patterns on the surfaces of bacteria. The alternative pathway is activated by the spontaneous hydrolysis of C3 in the blood, generating C3b that binds to bacterial surface molecules. All three pathways activate the complement cascade, a series of protein interactions that results in increased inflammation, innate immune activation, opsonization, and formation of the membrane attack complex (MAC). **B.** Mirror bacteria would trigger fewer pathways of complement activation. The classical pathway could be impaired due to lack of circulating IgG, but natural IgM antibodies or phosphocholine could activate C1q if present. The lectin pathway is unlikely to be activated because it depends on the recognition of chiral carbohydrates. The alternative pathway could remain functional because it involves no stereospecific interactions. Impaired complement activation might result in reduced inflammation, innate immune cell activation, opsonization, and formation of MACs.

In the classical pathway, circulating IgM or IgG antibodies bind to the surface of a pathogen and are subsequently bound by a protein complex called C1 (Mastellos *et al*., 2024). C1 (specifically the component protein C1q) then activates the complement cascade. Production of affinity-matured anti-mirror antibodies that could potently activate the classical pathway is expected to be absent or greatly reduced, but predominantly IgM antigen-specific antibodies might still be elicited through T cell-independent mechanisms (see Section 4.3). Natural IgM antibodies, which are present in circulation prior to antigen encounter and have not undergone affinity maturation to any specific antigen, typically have broad specificity and are able to weakly bind to a range of different antigens (Coutinho *et al*., 1995; Z.-H. Zhou *et al*., 2007). When bound to bacterial antigen, natural IgM is able to activate the classical complement pathway, albeit less potently than affinity-matured antibodies (Boes *et al*., 1998; Z.-H. Zhou *et al*., 2007). As such, natural IgM could plausibly play a role during early stages of mirror bacterial infection. Complement activation through natural IgM has been shown to confer resistance to bacterial infection in mice (Islam *et al*., 2023; Mold *et al*., 2002). However, it is uncertain, whether natural IgM would be able to bind mirror antigens, as some murine studies suggest that natural IgM polyreactivity is restricted to phylogenetically conserved microbial structures (Hardy & Hayakawa, 1994; Kantor & Herzenberg, 1993). If natural IgM antibodies indeed predominantly bind conserved epitopes, it would be less likely they would bind mirror versions of these epitopes. Without anti-mirror antibodies, the classical pathway would be largely unable to function.

The classical pathway can also be activated by C reactive protein (CRP) if it binds to phosphocholine in cell membranes and is subsequently bound by C1q (Thompson *et al*., 1999). However, although phosphocholine is achiral, it is absent from an estimated 85% of bacteria, including *E. coli* (Geiger *et al*., 2013; Kleetz *et al*., 2021). Moreover, even if phosphocholine were present on the surfaces of mirror bacteria, it is unclear whether CRP would be present at sufficiently high concentrations to alter the course of a mirror bacterial infection. Although CRP is constitutively produced by the liver, its concentration in the blood increases a thousand-fold during infection (from 1 µg/mL to 1000 µg/mL) in response to the inflammatory cytokines IL-6, IL1β, and TNF (Del Giudice & Gangestad, 2018; Ji *et al*., 2023). Both IL1β and TNF are primarily produced by activated macrophages (Ott *et al*., 2007), and as discussed in the previous subsection, it appears unlikely that significant macrophage activation would occur in response to mirror bacteria. IL-6, meanwhile, can be produced by epithelial cells in response to both MAMPs and DAMPs (Krueger *et al*., 1991), so it is possible that CRP concentrations could increase given sufficient inflammation during later stages of infection. If this occurred, a substantial amount of CRP might bind to phosphocholine (if present on mirror bacteria), and this could potentially preserve some functionality of the classical pathway. However, the upstream failures of pathogen recognition appear to be a significant obstacle to enhanced CRP production, and it is unclear whether low constitutive CRP levels would be sufficient to activate the classical pathway such that it would contribute to anti-mirror immunity in a meaningful way.

The alternative complement pathway could function against mirror bacteria because it is initiated by the spontaneous production of C3b, which is highly reactive and will covalently bond with any nucleophilic group (Law & Dodds, 1997). Factor B then binds to C3b and is cleaved to form a

C3bBb complex, which in turn can cleave C3 to form more C3b, so that the alternative complement activation can self-amplify (Merle *et al*., 2015). The C3bBb complex is short-lived, however, unless stabilized by the protein properdin, which is constitutively produced in blood plasma by leukocytes but also stored and released by neutrophils in response to inflammatory agonists (J. Y. Chen *et al*., 2018).

To summarize, complement activation would likely be impaired due to disruptions to both the lectin and classical activation pathway, but the alternative pathway could remain functional and this could allow some activation to occur. While many pathogens have evolved diverse strategies to avoid complement activation, such as attracting host complement regulators, enzymatically cleaving complement components, or mimicking host surfaces (Serruto *et al*., 2010), mirror bacteria would not by default have functional versions of these mechanisms. Therefore, one would expect C3b to opsonize mirror bacteria for phagocytosis, and for C3a and C5a (which are produced downstream of C3b) to be released and induce inflammation.

In the last stages of complement activation, C5b interacts with additional complement proteins to form a membrane attack complex (MAC) which is capable of rupturing Gram-negative cell envelopes (Doorduijn *et al*., 2019). This process is not known to depend on any specific surface receptors, so it is likely that the MAC could correctly assemble and target mirror Gram-negative bacteria. Gram-positive bacteria are resistant to lysis by MAC due to their thick peptidoglycan layer (Berends *et al*., 2013; Joiner *et al*., 1984).

It is difficult to estimate how the impairments in complement functioning described above would ultimately impact the ability of mirror bacteria to establish infection, especially in the context of other expected failures of the immune response. There are a number of inherited diseases of the human complement system that could be informative. If we assume that the alternative pathway would function during a mirror bacterial infection, but the lectin pathway and possibly the classical pathway would be impaired, then the most analogous human deficiency would be that caused by deficiencies in C2 or C4, which are shared by the latter two pathways (but not the alternative pathway). Both of these deficiencies are rare and incompletely characterized, but their primary clinical features are an increased susceptibility to bacterial infections, particularly to encapsulated bacteria. These features are broadly shared with other complement deficiencies, albeit with variations in autoimmune manifestations and to which bacterial species individuals are most susceptible (Pettigrew *et al*., 2009). Quantifying the degree of increased susceptibility to bacterial infection in C2 and C4 deficiencies is difficult given data scarcity.

Phagocytosis of mirror bacteria would likely be compromised

Phagocytosis is the process by which innate immune cells capture, kill, and clear bacteria (Gordon, 2016). The first phagocytic cells to encounter mirror bacteria would likely be tissue resident macrophages and dendritic cells. At rest, these cells exhibit a low level of phagocytosis, but they become highly phagocytic upon activation (Mosser & Edwards, 2008). As discussed above, macrophages and dendritic cells are unlikely to be activated by mirror MAMPs, thereby reducing the number of mirror bacteria these cells would attempt to phagocytose.

If macrophages fail to activate, this could also limit the number of neutrophils and monocytes that respond to mirror bacteria. Neutrophils, which play an essential role in the clearance of many bacteria as the most phagocytic innate immune cells, respond to inflammation by entering the tissues and then following gradients of cytokines, chemokines, and MAMPs to the infection site (Burn *et al*., 2021). Without these signals, many of which are produced by activated macrophages, neutrophils may face great difficulties in finding mirror bacteria. Like neutrophils, monocytes would also likely fail to respond to a mirror bacterial infection due to reduced inflammation and impaired ability to sense MAMPs. This could hinder both entry into infected tissues and differentiation into new macrophages and dendritic cells (Jakubzick *et al*., 2017).

Overall, we expect few phagocytes to respond to mirror bacteria at the beginning of an infection. This could change at later points if the release of DAMPs from necrotic cells combined with non-specific inflammation is sufficient to activate macrophages and dendritic cells and recruit neutrophils and monocytes to the infection site. Accumulation of the soluble complement factor C5a, which can induce inflammation, activate phagocytes, and promote neutrophil chemotaxis, may also improve the likelihood of a phagocyte response (Guo & Ward, 2005), although it is unclear how these functions would be affected by the lack of PRR signaling.

Regardless of their activation status, the reversed chirality of mirror bacteria would likely make it difficult for phagocytes to internalize them. Phagocytosis is an active, receptor-mediated process. Phagocytic receptors bind either directly to bacteria or indirectly via opsonizing complement proteins or antibodies bound to bacteria. These receptors enable phagocytes to grasp the bacterial cells and, importantly, activate signaling pathways that induce the cytoskeletal changes required for internalization (Uribe-Querol & Rosales, 2020). Most phagocytosis receptors are not expected to bind mirror bacteria due to the reversed chirality of their ligands. Even if some receptors did bind, this would not necessarily enable phagocytosis if PRRs or other signals are absent. Scavenger receptors, for instance, have broad specificities and may in theory bind some mirror molecules. But they generally partner with PRRs or Fc receptors to signal (Alquraini & El Khoury, 2020; Fu & Harrison, 2021), and therefore may be unable to facilitate phagocytosis by themselves. Complement receptors would also still bind complement-opsonized mirror bacteria without additional assistance, which might allow some mirror bacteria to be internalized. Again, however, this process would plausibly be less efficient because the affinity of complement receptors for their ligands is typically enhanced by signaling from other phagocytic receptors and PRRs (Uribe-Querol & Rosales, 2020). Macrophages can internalize inanimate particles like latex beads (Collin-Faure *et al*., 2023) or asbestos (Ishida *et al*., 2019), suggesting some nonspecific uptake of mirror bacteria may also occur. Still, the overall reduced functionality of phagocytic receptors suggests mirror bacteria could be internalized at lower rates than comparable non-mirror bacteria.

It is also unclear whether phagocytosis would kill those mirror bacteria that are internalized. Once engulfed, mirror bacteria would be enveloped in a new vesicle called the phagosome, which would eventually mature via fusion with lysosomes to form a bactericidal organelle called the phagolysosome (Sivaloganathan & Brynildsen, 2021). Phagosomes containing synthetic beads can mature into phagolysosomes even in resting phagocytes (Alloatti *et al*., 2015; Dill *et al*., 2015; Hoffmann *et al*., 2012; Sanjuan *et al*., 2007), suggesting internalized mirror bacteria would also

arrive in phagolysosomes, although the absence of MAMPs or inflammatory cytokines might speed or slow phagosome maturation, depending on the context (Pauwels *et al*., 2017).

Normally, bacteria are killed in phagolysosomes by oxidants that permeabilize the cell membrane, AMPs, or activity of antimicrobial enzymes whose function is enhanced by low phagolysosomal pH (~4.5) (Brown, 2024). As discussed earlier, AMPs that disrupt membranes via electrostatic and amphipathic interactions would likely be unaffected by the reversed chirality of mirror bacteria. Because many of them are constitutively expressed in the lysosomes of phagocytes (M. S. Park *et al*., 2018), they may also be present at concentrations high enough to damage mirror bacteria. Antimicrobial enzymes, however, would likely not function against mirror bacteria due to incompatible chirality. In addition, many kinds of mirror bacteria, including mirror *E. coli*, could potentially survive the phagolysosomal acidity, given that many natural bacteria can survive in acidic conditions with pH < 4.5 (Baka *et al*., 2013; Gorden & Small, 1993; Ross *et al*., 2003).

On the other hand, oxidants like reactive oxygen species (ROS) would remain harmful to mirror bacteria. Although the exact mechanism by which ROS contributes to antibacterial immunity is controversial (Herb & Schramm, 2021), there is some evidence that ROS are sufficient to kill bacteria in phagolysosomes. A recent study shows that the ROS that accumulate in phagocytic vesicles immediately after internalization can kill *Pseudomonas aeruginosa* cells without the aid of antimicrobial enzymes in murine alveolar macrophages (Riazanski *et al*., 2020). It is not known whether this can be generalized to other phagocytic cells or to other species of bacteria, or whether the oxidant levels in the phagolysosomes of unactivated phagocytes would be sufficient to kill mirror bacteria. Moreover, mirror bacteria could plausibly be protected from low levels of ROS (which are achiral) by the same catalases that naturally protect *E. coli* from oxidative stress (Khademian & Imlay, 2021; Sultana *et al*., 2022). Thus, it is difficult to determine how effective ROS would be at killing internalized mirror bacteria by themselves. If ROS levels were low enough, it is plausible that many mirror bacteria would survive.

To summarize, the available evidence suggests phagocytosis of mirror bacteria would be less effective compared to phagocytosis of natural bacteria (Figure 4.4). Phagocytes may have difficulty finding mirror bacteria, engulfing them, and killing them. Some mirror bacteria may survive inside phagocytes. Furthermore, even those that are killed would not be properly digested due to incompatibilities between lysosomal enzymes and mirror macromolecules. In either case, phagosome resolution—the process by which phagocytes digest and recycle the contents of phagolysosomes (Mylvaganam & Freeman, 2023)—would probably be disrupted.

While the persistence of live mirror bacteria and mirror bacterial debris inside phagocytes seems likely to impede their function, the implications are unclear. During infections with natural-chirality bacteria, the presence of live intracellular bacteria or intracellular MAMPs can trigger programmed cell death, often in the form of apoptosis, pyroptosis, or necroptosis, which enclose the pathogen in cellular bodies that are processed by phagocytes (Lacey & Miao, 2020). Pyroptosis and necroptosis additionally induce broad-spectrum inflammatory responses that can aid in clearing bacterial

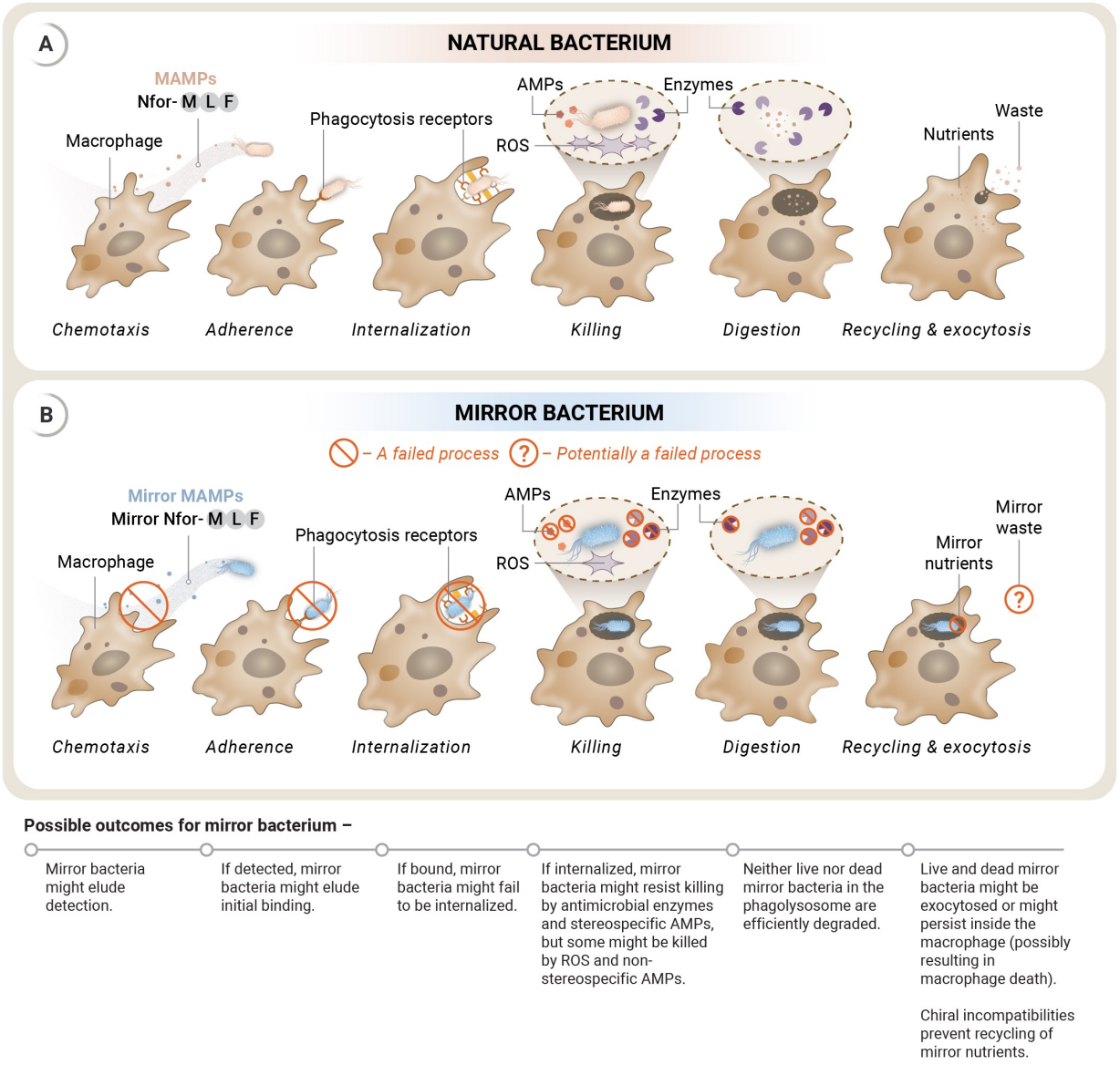

Figure 4.4: Mirror bacteria could evade phagocytosis and killing by macrophages
A. Natural bacteria trigger a number of processes that enable macrophages to phagocytose and kill them. Macrophages detect microbe associated molecular patterns (MAMPs), including formyl-peptides (Nfor-MLF) and chemotax toward bacteria. Phagocytosis receptors bind bacteria and initiate their internalization. After internalization, phagolysosomal reactive oxygen species (ROS), antimicrobial peptides (AMPs), and enzymes kill phagocytosed bacteria, which are then digested, their nutrients being recycled and waste products exocytosed. **B.** Mirror bacteria would not be expected to induce chemotaxis, binding, or substantial internalization by macrophages because each of these processes relies on macrophage receptors binding chiral proteins or peptides. If a mirror bacterium is internalized, it may or may not be killed in the phagolysosome, as some mechanisms of cell lysis are stereospecific (antimicrobial enzymes, some AMPs) but others (ROS, potentially other AMPs) are not. Digestion of mirror bacteria and nutrient recycling are unlikely to function properly, potentially resulting in macrophage death or decreased function.

infections, but they can also harm the host by continuously releasing DAMPs if not properly regulated (Booty & Bryant, 2022). However, intracellular mirror bacteria might not trigger programmed cell death. Pyroptosis and necroptosis are often initiated through MAMP detection by intracellular PRRs—e.g., TLRs or NLRs (Jorgensen *et al*., 2017). Necroptosis can also be triggered directly by inflammatory cytokines or bacterial virulence factors (X. Yu *et al*., 2024), whereas pyroptosis can be initiated by specialized host proteins that detect disruptions in cellular pathways (Lacey & Miao, 2020). Mirror bacteria would likely be resistant to PRR binding, would not have functional virulence factors to directly trigger cell death, and it is unclear whether cellular pathways would be sufficiently disrupted by the persistence of mirror bacteria in phagocytic vesicles. Taken together, it is plausible that mirror bacteria would avoid triggering programmed cell death unless persistence of live bacteria or debris severely interferes with cellular pathways. Ultimately, even if triggered, programmed cell death would likely not facilitate clearance, as the phagocytes taking up the cellular bodies containing mirror bacteria would in turn face difficulties lysing them.

The role of innate lymphoid cells in mirror bacterial infection is unclear

Innate lymphoid cells (ILCs)—specifically group three ILCs (ILC3s)—are one additional innate immune cell type known to be activated during bacterial infections. ILC3s are found in lymphoid tissues and near barrier surfaces such as the gut, respiratory tract, and skin (Sonnenberg & Hepworth, 2019) and are an early source of effector cytokines that elicit and support antibacterial immune responses in helper T cells and other cell types (Vivier *et al*., 2018; von Burg *et al*., 2014). Most pathways of ILC3 activation seem likely to be impaired in the case of mirror bacteria. The primary drivers of ILC3 activation are inflammatory cytokines secreted by activated phagocytes (W. Zhou & Sonnenberg, 2020), but this pathway may be hindered by the impaired activation of these cells during mirror bacterial infection. Human ILC3s also express TLRs and can be directly activated by MAMPs, but, as discussed earlier, TLR signaling is likely to be significantly impaired in the case of most mirror MAMPs. Although ILC3s can be directly activated by bacterial tryptophan metabolites and components of bacterial cell walls (W. Zhou & Sonnenberg, 2020), these are all chiral and unlikely to be sensed in their mirror form. ILC3s can also directly sense short chain fatty acids (W. Zhou & Sonnenberg, 2020), some of which are achiral, such as acetic acid, propionic acid, and butyric acid, and would likely be sensed if they were secreted by mirror bacteria. The overall impact of ILC3s sensing mirror bacteria via short chain fatty acids is unclear because experiments show that this pathway can either promote or suppress the production of effector cytokines depending on the specific location and receptors involved (Chun *et al*., 2019; S.-H. Kim *et al*., 2017). Moreover, short chain fatty acids are produced in abundance by commensal bacteria (Fusco *et al*., 2023), so it is not obvious that their production by mirror bacteria would alter ILC3 function. Thus, although ILC3s can promote antibacterial responses, whether they would be relevant during a mirror bacterial infection is unclear.

4.3 Adaptive immunity to mirror bacteria would likely be impaired

In addition to the innate immune system, humans also possess an adaptive immune system. A key difference between the innate and adaptive systems is that while the innate immune system relies on

invariant PRRs for pathogen detection, the adaptive immune system uses a process of genetic recombination to generate a large and diverse repertoire of variable receptors, each capable of binding to different pathogen-derived molecules. This vast repertoire of adaptive immune receptors can bind a wide array of biological macromolecules (Chiu *et al*., 2019; Marks & Deane, 2020) and could bind mirror macromolecules. However, B or T cell receptor binding is only one of the components necessary for B and T cell activation. As we describe in the following section, T and B cell activation also depends on a diversity of signals from the innate immune system and, in the majority of cases, the successful presentation of peptide antigens. We expect these to be impaired, and thus we do not expect humans to mount a strong adaptive immune response to mirror bacteria. The likelihood of a weak adaptive immune response would mean the formation of protective memory responses may also be impaired.

T cell activation would likely be severely impaired during mirror bacterial infection

The activation of conventional T cells requires a number of steps, most of which could have serious defects in response to a mirror bacterial infection. Adaptive immunity is initiated by dendritic cells from the innate immune system (Iwasaki & Medzhitov, 2015). In a bacterial infection, dendritic cells that have been activated via PRR stimulation increase their uptake of bacteria and then migrate to lymph nodes. There, the dendritic cells present peptides from the bacteria to naive T cells. If there are T cells with TCRs that can recognize the bacterial peptides, they become activated and proliferate, effectively initiating the adaptive immune response. Activated T cells either migrate to the infected tissues, where they kill infected cells and secrete proinflammatory cytokines, or they remain in the lymph node and activate B cells, enabling the latter to make antibodies.

The inability of the innate immune system to detect most, if not all, mirror MAMPs would likely prevent dendritic cells from being activated. This would result in few, if any, dendritic cells trafficking to the lymph nodes and being available to activate T cells—presenting a substantial barrier to mounting an adaptive immune response.

Even if dendritic cells were activated in large numbers, able to phagocytose mirror bacteria, and subsequently migrate to the lymph node, it appears unlikely that they would be able to efficiently process and present mirror peptide antigens to T cells. Antigen presentation involves multiple stereospecific steps (Figure 4.5, left-hand side). In a normal immune response to extracellular bacteria, bacteria-derived proteins taken up by dendritic cells are first cleaved by lysosomal proteases into short (13-25 amino acid) peptides before binding to major histocompatibility complex class II molecules (MHC-II)[8] (Chicz *et al*., 1992). Peptide:MHC-II complexes on the surface of dendritic cells are presented to the TCRs of naive T cells expressing CD4, a co-receptor for MHC-II. Each $CD4^+$ T cell bears a unique TCR sequence, endowing it with a distinct structure and the ability to bind specifically to a narrow set of peptide:MHC-II complexes. If this binding takes place with high affinity (and proper co-stimulation through other, non-pathogen-specific receptors expressed on both

[8] We focus on MHC-II because we expect mirror bacterial infection to be largely extracellular. Although antigen presentation by MHC-I would suffer analogous defects to MHC-II, this would likely be less relevant during mirror bacterial infection because MHC-I molecules typically present peptides derived from intracellular pathogens.

cells), the T cell is activated, allowing it to proliferate. Each stage of this process would likely face substantial challenges during a mirror bacterial infection.

As discussed in Section 4.2, mirror proteins and mirror peptides resist protease degradation. With respect to antigen processing, an inability to degrade mirror proteins has been demonstrated for two lysosomal proteases that process peptides for MHC-II molecules (Chu *et al*., 2012; Meldal *et al*., 1998). Given the available evidence, it appears unlikely that antigen-presenting cells would successfully cleave the vast majority of mirror peptides derived from mirror bacteria, generating few, if any, mirror peptides as candidates for binding to MHC molecules (Figure 4.5, Step 1). This would substantially reduce the ability of dendritic cells to activate T cells. However, it is possible that small peptides could be derived from lysed mirror bacteria directly, as mirror bacterial proteases would be able to cleave mirror proteins.

Even if mirror peptides were available to bind MHC molecules, it seems likely that this step would be relatively inefficient. Mirror peptides of appropriate size and amino acid composition (*vis a vis* regular peptides) tend to bind MHC molecules only weakly, if at all (Bartnes *et al*., 1997; Hervé *et al*., 1997; Hill *et al*., 1994; Lombardi *et al*., 2020; Mézière *et al*., 1997). Thus, during a mirror bacterial infection, only a fraction of the available mirror peptides may be expected to bind MHC molecules with sufficient affinity to be presented on the surfaces of dendritic cells in peptide:MHC complexes (Figure 4.5, Steps 2 and 3).

Were mirror-bacteria-derived, high-affinity mirror peptide:MHC complexes to be presented to naive T cells, the extent to which T cells would be activated is unclear. TCR binding to a mirror peptide:MHC complex can involve interactions between the TCR, MHC molecule and both the side chains and backbone of the peptide being presented (Rossjohn *et al*., 2015) (Figure 4.5, Step 4 and 5). The enormous diversity of TCR receptors and MHC allotypes, with a genetic recombination process in each cell that is capable generating one of 10^{18} potential TCRs (Sethna *et al*., 2019) and with more than 40 000 MHC alleles in the human population (encoding over 26 000 distinct protein sequences) (EBI Web Services, n.d.), provides many potential combinations that could result in functional TCR-peptide-MHC interactions (Heijmans *et al*., 2020). The available experimental evidence shows that T cells can respond to mirror-peptide:MHC complexes, but the response is generally weaker than to similar peptide:MHC complexes (Bartnes *et al*., 1997; Chong *et al*., 1996; Mézière *et al*., 1997; Nair *et al*., 2003). Very few peptides have been tested, however, so it is not clear if this is because D-peptides are less able to activate T cells or to be efficiently presented by MHC, or if this is simply an artifact of the handful of examples studied in the literature.

In addition to engagement of the TCR with an MHC-peptide complex, T cells need additional signals to become fully activated, including binding of dendritic cell co-stimulatory molecules CD80 or CD86 to T cell molecule CD28, and activating soluble factors like IL-6, IL-12, or IL-23. These activation factors are largely regulated by PRR signaling, and plausibly would not be significantly upregulated during a mirror bacterial infection (Figure 4.5, Step 6). As such, T cells that can recognize mirror bacterial peptides might not receive sufficient costimulatory signals to survive and differentiate into effector T cells.

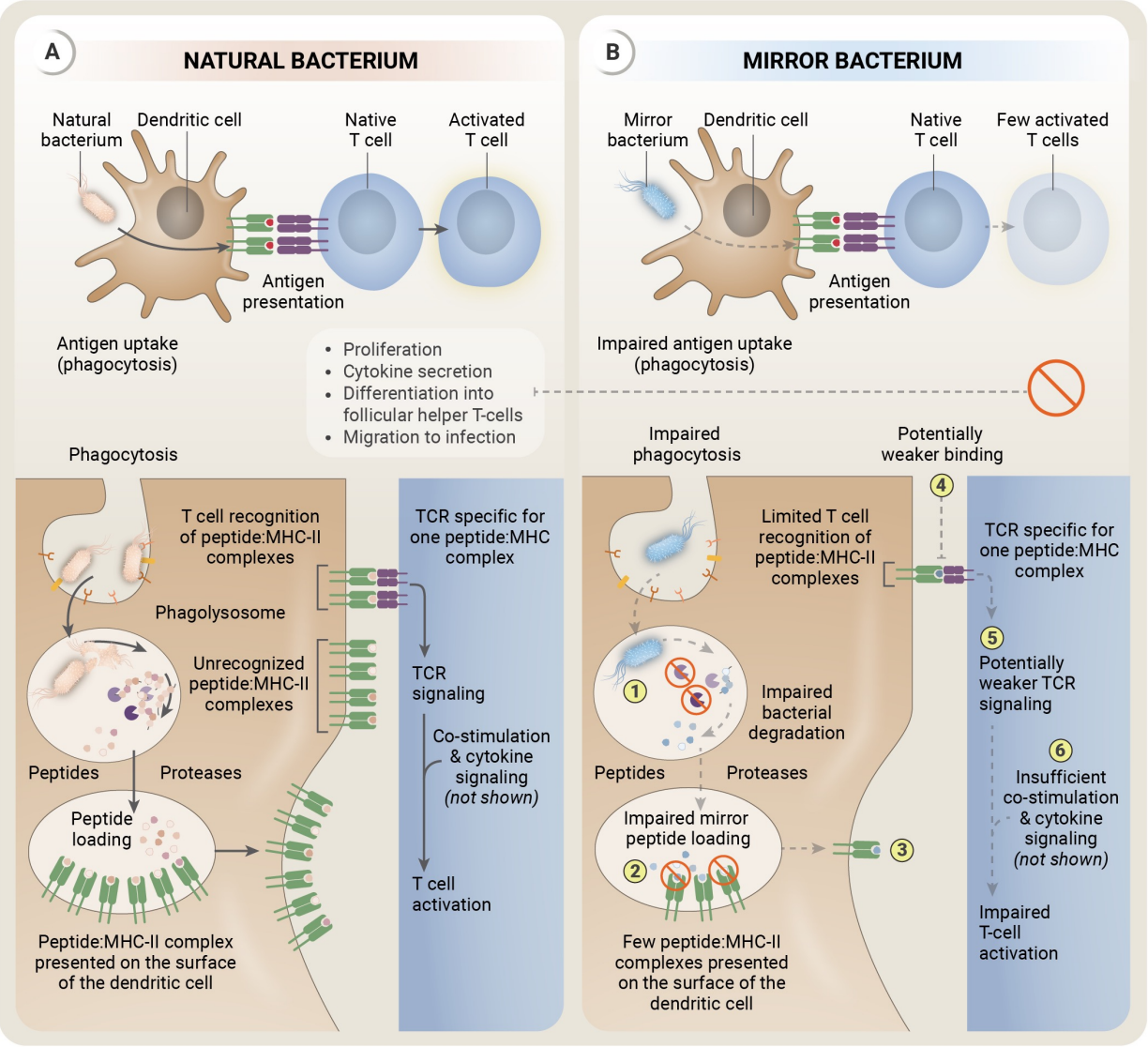

Figure 4.5: T cell activation is likely to be severely impaired in response to mirror bacterial infection
A. T cell activation in response to natural-chirality bacteria is a multi-step process. Activated dendritic cells phagocytose bacteria and degrade their proteins into peptides, many of which are loaded onto major histocompatibility complex (MHC) molecules, forming peptide:MHC complexes that translocate to the cell surface. Peptide:MHC complexes are recognized by T cell receptors (TCRs), resulting in T cell activation. **B.** Many steps of T cell activation could be impaired in responses to mirror bacteria. Mirror bacteria would likely evade phagocytosis by dendritic cells because this action is mediated by stereospecific protein receptors. If mirror bacteria were phagocytosed, the proteases that mediate protein degradation and peptide formation are stereospecific as well, so few mirror peptides would likely be produced (Step 1). If mirror peptides were formed, MHC molecules would likely bind them weakly, if at all (Step 2) and few mirror peptide:MHC complexes would be presented on the surface of the dendritic cell (Step 3). Existing data, although limited, suggests that TCRs might bind mirror peptide:MHC complexes weakly (Step 4). Failure of these steps could weaken TCR signaling (Step 5). Impaired innate immune activation could also result in insufficient co-stimulation and cytokine signaling necessary for T cell proliferation (Step 6). Taken together, T cell activation in response to mirror bacteria would likely be severely impaired.

The combined effect of impaired innate immune activation, protease resistance, weak MHC binding of most mirror peptides, and (potentially) weak T cell recognition is likely to severely impair T cell activation (Figure 4.5). Although it is hard to rule out the possibility that a small subset of mirror peptides could pass through the aforementioned hurdles—the space of mirror peptides is very large—this seems significantly less likely to occur during a mirror bacterial infection compared to a natural bacterial infection. It therefore appears that a mirror bacterial infection could result in a diminished or ineffective T cell response.

Antibacterial responses in peripheral tissues are enhanced by the functions of various types of T cells and other lymphoid cells. Therefore, impaired activation of these cells could weaken the overall immune response to mirror bacteria. Activated helper T cells—particularly Th17 cells—are important for driving a potent antimicrobial response. During infections with natural extracellular bacteria, activated dendritic cells secrete cytokines that cause many newly activated T cells to differentiate into Th17 and other helper T cells (Korn *et al*., 2009). These T cells then migrate to the site of infection and secrete cytokines that greatly enhance neutrophil recruitment and cause epithelial cells to proliferate, increase the formation and maintenance of tight junctions, and secrete antimicrobial peptides (Lewinsohn *et al*., 2011; Mills, 2023; Schnell *et al*., 2023; Wan & Flavell, 2009). Impaired generation of helper T cells during a mirror bacterial infection could diminish these antibacterial mechanisms, including AMP production, mucus secretion, enhancement of tight junctions, and transport of IgM and IgA across epithelial barriers (Mills, 2023), all of which might otherwise be effective against mirror bacteria. On the other hand, any damage caused by a mirror bacterial infection could expose innate immune cells to MAMPs from natural bacteria (especially at mucosal surfaces like the gut), causing them to produce inflammatory mediators that would stimulate tissue resident helper T cells to produce inflammatory cytokines and enhance the local innate immune response.

CD1- and MR1-restricted T cells might be able to recognize some mirror ligands

CD1-restricted and MR1-restricted T cells secrete potent inflammatory cytokines upon activation, which can be protective against many bacterial species (Mori *et al*., 2016). It is possible that both cell types would be able to detect some mirror bacterial ligands and activate during a mirror bacterial infection.

CD1-restricted T cells detect microbial lipids from a variety of bacterial species (e.g., Bacteroides and Mycobacteria) presented by antigen presenting cells via the nonclassical MHC molecules CD1a, CD1b, CD1c, and CD1d (Van Rhijn *et al*., 2015). Although CD1 molecules have limited sequence variation (Salio *et al*., 2014), the mechanism by which they bind their ligands does allow for some flexibility in the lipid antigens they present. CD1 molecules have deep, hydrophobic pockets that bind the largely achiral alkyl chains of microbial lipids, which allow the chiral head regions of the lipids to project up out of the binding pocket and interact with TCRs (Gras *et al*., 2018). Although the loading of glycolipids into CD1 molecules can require chirality-dependent processing by host lipases and glycosidases, smaller lipids can be loaded without the need for accessory proteins (Salio *et al*., 2014). This mechanism allows CD1 molecules to bind a limited diversity of lipid antigens and might also permit CD1 molecules to bind and present some mirror lipids. Moreover, although the TCRs of

CD1-restricted T cells are less variable than those of conventional T cells, they maintain some variation, which might enable them to bind to the achiral regions of mirror lipids. Together, these characteristics suggest that some CD1-restricted T cells might be activated during a mirror bacterial infection.

MR1-restricted T cells share many characteristics of CD1-restricted T cells, but recognize small molecule metabolites instead of lipids. These metabolites are released by live bacteria and bind to MR1, a nonclassical MHC molecule expressed by a broad range of cell types. Although MR1 displays limited sequence variation in humans (Rozemuller *et al*., 2021) and all known stimulatory MR1 ligands are chiral, the molecule has a flexible binding pocket that might accommodate mirror bacterial metabolites (McWilliam & Villadangos, 2024). Further study may reveal additional, achiral ligands. In addition, MR1 ligands are not derived from larger precursor molecules, and therefore could plausibly be available in the phagolysosome without the need for enzymatic processing (Mori *et al*., 2016). Although the TCRs of MR1-restricted T cells are semi-invariant, the presence of some sequence variation makes it difficult to rule out the recognition of mirror metabolites presented by MR1. Thus, similar to the case for CD1-restricted T cells, it seems possible that some MR1-restricted T cells could be activated by mirror bacteria.

Ultimately, the extent to which CD1- and MR1-restricted T cell activation would enhance immune responses to mirror bacteria is unclear. Most bacterial infection studies rely on mouse models, but mice make poor models to study these cells: they have much lower percentages of MR1-restricted T cells than humans, and they lack CD1a, CD1b, and CD1c (Mori *et al*., 2016). In addition, not all bacteria express (known) CD1 or MR1 ligands. As such, it is difficult to predict to what extent CD1- and MR1- restricted T cells would contribute to protection against mirror bacteria.

Impaired T cell activation would impair antibody-mediated immunity to mirror bacteria

Antibodies typically play an important role in immune responses to extracellular bacteria by blocking bacterial binding to host surfaces, opsonizing bacterial cells for phagocytosis, and neutralizing bacterial toxins (Lu *et al*., 2018). Antibodies against protein antigens are normally generated after B cells in secondary lymphoid organs capture the proteins via their B cell receptors (BCRs), internalize them, and subsequently process and present peptide:MHC-II complexes to activated helper T cells. The T cells that recognize the peptide:MHC-II complex then deliver activating signals to that B cell, enabling it to proliferate and produce soluble antibodies. While B cells are expected to have BCRs that could recognize mirror proteins, their ability to process and present mirror peptides would be impaired. Impaired ability to process and present mirror peptides would severely limit B cell survival, differentiation potential, and the potential for these BCRs to undergo affinity maturation in secondary lymphoid organs because all of these processes rely on (sometimes repeated) T cell help (Victora & Nussenzweig, 2022). Even if some mirror peptides were presented, the previously described deficit of activated T cells could leave the corresponding B cells without T cell help. Instead of proliferating and maturing, these B cells would then become anergic or undergo apoptosis (Turner *et al*., 2020; Y. Wang *et al*., 2020; Yarkoni *et al*., 2010). The lack of T cell help could also reduce the B cell response to non-protein mirror antigens. Non-protein antigens from bacteria are often associated with proteins as part of intact cells or cell fragments (Kappler & Hennet, 2020). B

cells with BCRs that bind non-protein antigens thus internalize proteins as well, and these proteins are subsequently presented via peptide:MHC-II complexes to helper T cells, which, in turn, activate the B cells to produce antibodies against the non-protein antigen. Impaired T cell-dependent B cell activation could therefore result in a severe reduction in the overall antibody response to mirror bacteria.

B cells can also be activated without helper T cells, but it is unclear how effective this would be in response to mirror bacteria. A major activation mechanism requires the crosslinking of a sufficient number of BCRs by highly repetitive molecules, such as the capsular polysaccharides found on the surfaces of encapsulated bacteria (Vos *et al*., 2000). Such crosslinking could plausibly be triggered by mirror bacteria, particularly by mirror encapsulated bacteria. While crosslinking of BCRs alone can lead to B cell proliferation (Snapper, 2016), T cell-independent antibody secretion generally requires co-stimulation with TLR ligands, although other co-stimulation routes have been documented, especially those involving IL-2 and IL-5 (Snapper, 2016). The extent of co-stimulation that would occur in response to mirror bacteria remains unclear. In particular, we expect TLR stimulation by most mirror MAMPs to be impaired, although it is possible that yet unseen co-stimulation routes would emerge. Thus, it is possible, though uncertain, that plasmablasts secreting IgM antibodies against mirror bacteria may arise, which would offer an avenue for the activation of the classical complement pathway (see Section 4.2).

It is unclear to what extent T cell-independent B cell activation would be able to elicit IgG antibodies directed toward mirror bacteria. Although class switch recombination and AID activity have been observed in T cell independent mechanisms, researchers had previously observed that a second signal provided via pathways other than the BCR, such as TLR co-stimulation, is typically necessary for these processes (Pone *et al*., 2012; Snapper, 2016). A reduction in IgG antibodies would, at a minimum, result in a functionally altered response; IgG antibodies are typically the most abundant antibodies in serum, and can activate innate immune cells via Fc receptors and initiate the complement cascade via the classical pathway (Lu *et al*., 2018). Additionally, IgGs can penetrate extravascular sites and provide signaling through Fc receptors that enhance the phagocytic efficiency of phagocytes (Uribe-Querol & Rosales, 2020).

Although we expect the production of mirror-antigen-specific IgG antibodies would be impaired, B cells in the gut may be able to produce IgA antibodies upon encountering mirror bacteria in the gut. Chronic immune stimulation from the intestinal microbiota creates an environment that promotes the activation of B cells whose BCRs bind to T cell independent antigens (e.g., polysaccharides), allowing them to undergo isotype switching and differentiate into plasma cells that produce IgA in gut associated lymphoid tissues (GALT) (Bemark *et al*., 2024; Fagarasan *et al*., 2010). IgA antibodies are the dominant isotype in the gut and are particularly effective at trapping bacteria in the mucus layer and keeping them from adhering to epithelial cells (T. Chen *et al*., 2020). Research has also suggested that IgA antibodies against T cell independent antigens can acquire mutations through mechanisms that are not well-defined, allowing them to undergo affinity maturation (Bemark *et al*., 2024). Together, this suggests that GALT-resident B cells responding to mirror bacteria in the gut might generate IgA antibodies against mirror antigens. Furthermore, if γδ T cells in the GALT become activated by DAMPs, cytokines, and/or the achiral MAMP HMBPP, this may increase the

likelihood of anti-mirror IgA antibodies production, as γδ T cells have been proposed to directly interact with B cells in the GALT to activate them (Rampoldi *et al*., 2020). Even if these mechanisms do not enable GALT-associated B cells to produce IgA antibodies specific for mirror antigens, high concentrations of IgA antibodies are typically present in the gut, and many bind to specific gut bacterial species (C. Yang *et al*., 2022). Therefore, it is plausible that some may have cross-reactivity with mirror bacterial epitopes.

Some individuals might fortuitously have pre-existing antibodies that would cross-react with mirror ligands. These could be natural IgM antibodies which, as discussed in Section 4.2, may have broader specificities than other antibody types. Alternatively, they could be antibodies previously produced in response to other pathogenic infections that happen to cross-react with mirror antigens. Natural IgM antibodies would be able to initiate the classical complement pathway, but would not be able to directly opsonize mirror bacteria because the Fc receptor for IgM (FcμR) is not expressed on phagocytic cells (Kubagawa *et al*., 2009). For cross-reactivity with antibodies from previous infections, mirror antigens able to crosslink the BCRs of memory B cells would likely be sufficient to induce an antibody response. However, as discussed above, impaired T cell responses may hamper the strengthening of this humoral response via B cell differentiation or the affinity maturation of mirror-specific antibodies (Snapper, 2016).

Experimental evidence shows that mice immunized with full-length mirror proteins do not produce detectable IgG, despite producing robust IgG responses to non-mirror versions of the same proteins (King *et al*., 1994; Marinec *et al*., 2021; Uppalapati *et al*., 2016). Although co-stimulation with an adjuvant containing natural-chirality MAMPs did result in some IgG production against a mirror protein in one study (Dintzis *et al*., 1993), this mechanism is unlikely to be relevant for mirror bacterial infections due to the lack of natural-chirality MAMPs. We are not aware of any studies examining IgM or IgA responses to mirror proteins. As discussed above, failure to produce antibodies against mirror proteins is likely due to their resistance to proteolytic processing into peptides suitable for MHC loading. Since a majority of protein antigens derived from mirror bacteria are likely to exist as full-length folded proteins, the results of these studies are consistent with a dramatically impaired antibody response to mirror bacteria.

In contrast to full-length mirror proteins, immunization with short (7–26 amino acid) mirror peptides has been shown to produce weak or altered IgG responses compared to non-mirror peptide controls in laboratory animals (Arranz-Gibert *et al*., 2018; Chong *et al*., 1996). However, these mirror peptide studies may be less relevant to mirror bacterial infection because they involved the injection of concentrated doses of a single peptide species along with adjuvants containing MAMPs, rather than intact proteins in the absence of MAMPs.

Several studies published between 1960 and 1993 have been interpreted as evidence that mirror peptides are immunogenic (Sela & Zisman, 1997; Van Regenmortel & Muller, 1998), but the relevance of these studies to mirror bacteria is unclear for multiple reasons. In three studies, animals were immunized with long, highly repetitive mirror peptides (Gill *et al*., 1964, 1967; Janeway & Sela, 1967) that are unlike the peptides expected to exist in a mirror bacterium. Furthermore, in two of these two studies, the peptides also contained 1–4% L-amino acids, which could have contributed to the observed immune response (Jung, 1992; Stupp & Sela, 1967). Several other contemporaneous

studies with similar peptides failed to detect antibody responses (Borek *et al*., 1965; Gill *et al*., 1963; Maurer, 1963, 1965). Other studies employed mirrored versions of branched multichain peptides—which are not found in nature—as immunogens (Mozes *et al*., 1974; Sela *et al*., 1972; Simonian *et al*., 1968; Zisman *et al*., 1993). In another two studies cited as evidence for the immunogenicity of mirror peptides, animals were immunized with mirror peptides conjugated to liposomes filled with MPLA, a potent TLR agonist that may have elicited T-independent antibody responses (Benkirane *et al*., 1993; Guichard *et al*., 1994). Moreover, some of these studies indicated mirror peptides were only immunogenic when administered at low doses (Gill *et al*., 1964; Janeway & Sela, 1967; Mozes *et al*., 1974). The experimental design of these early studies and their lack of replication since publication more than 50 years ago highlight the urgent need for further research in this area.

In summary, secretion of mirror bacteria-specific antibodies would likely rely almost exclusively on T cell-independent routes. Although T cell-independent routes have been known to elicit antibody secretion and even durable antibody memory—for example, the Pneumovax-23 vaccine (Grabenstein & Manoff, 2012)—they typically require co-stimulation from other immune pathways, notably those involving TLRs. Given the expected impaired activation of the innate immune system, it is less clear where co-stimulating signals may come from. Moreover, additional signals are often critical in determining the functional form of an antibody response, including the isotypes of antibodies that eventually arise. The unusual features of mirror bacterial infection make it very difficult to anticipate the functional features of antibody responses against mirror bacteria.

Immune memory responses formed after an initial infection enable faster, more effective responses against future exposures to the same pathogen and are less dependent on MAMP and DAMP signaling (Chandok & Farber, 2004; Lam *et al*., 2024; Palm & Henry, 2019). However, durable memory T cell and B cell responses are dependent on a robust adaptive immune response to the initial infection (Palm & Henry, 2019; Pennock *et al*., 2013). Therefore, the impaired initial immune response described here would likely preclude the formation of protective memory responses to mirror bacteria as well.

Mirror bacteria evasion of adaptive immunity might mimic MHC-II immune deficiency

MHC-II deficiency is likely the closest clinical analog to the severe deficit in antigen presentation expected during mirror bacterial infections. MHC-II deficiency, also known as bare lymphocyte syndrome type 2, is a rare autosomal recessive primary immunodeficiency where loss-of-function in transcription factors for MHC-II results in reduced or absent expression of MHC class II molecules on the surface of immune cells (Reith *et al*., 2013). This condition results in a profound impairment of antigen-specific T and B cell responses because of MHC-II's essential role in antigen presentation.

Without stem cell transplantation—the only curative treatment—the prognosis for MHC-II deficiency is bleak. Despite supportive care, antimicrobial prophylaxis, and immunoglobulin administration, the typical clinical course involves multiple severe acute and chronic infections, and death typically occurs within the first decade of life (Hanna & Etzioni, 2014; Reith *et al*., 2013).

Survival into adulthood is rarely documented, and the factors that explain these more favorable outcomes are poorly understood.

Although a close mechanistic analog, MHC-II deficiency would not exactly match the severe deficits in antigen presentation expected for healthy individuals challenged with mirror bacteria. Complete absence of response to foreign antigens is not the only immunological feature of MHC-II deficiency: most patients have a substantial CD4+ T-cell cytopenia, likely due to the requirement of MHC-II for T-cell maturation, and 20% of patients have autoimmune manifestations. Although the deficit in antigen presentation is likely the primary driver of the immunodeficiency—successful stem cell transplant normalizes antigen responses while CD4+ T cell counts remain low (Ouederni *et al*., 2011), and autoimmune manifestations do not correlate with prognosis (Hanna & Etzioni, 2014)—these additional immunological defects associated with MHC-II deficiency may contribute to a greater overall functional deficit in immunity than would occur in a healthy immune system challenged with mirror bacteria.

The poor outcomes of MHC-II deficiency are attributable to a broad and pervasive vulnerability to infection from many organisms, not just bacteria. Alongside bacterial infections and bacterial sepsis, viral meningitis, liver failure secondary to infection by protist *Cryptosporidium parvum*, and pneumonia caused by the fungus *Pneumocystis jiroveci* are life-threatening complications frequently reported in MHC-II deficiency case series (Ben-Mustapha et al., 2013; Ouederni et al., 2011). Nevertheless, MHC-II deficiency demonstrates a mechanistically similar deficit to that expected against mirror bacteria and is sufficient to confer extreme vulnerability to bacterial infection. In one case series of 35 patients with MHC-II deficiency (Ouederni et al., 2011), 11 (31%) had documented episodes of bacterial sepsis, 10 (28%) bacterial pneumonia, and 5 (14%) bacterial pyelonephritis, numbers which are greatly elevated compared to the general population (e.g., the global incidence of pediatric and neonatal sepsis is estimated to be 48 cases per 100 000 person years (Fleischmann-Struzek et al., 2018). This vulnerability to bacterial disease may still be underestimated in the MHC-II clinical data due to extensive anti-bacterial prophylaxis (which may not be easily available for infections with mirror bacteria, see Chapter 5), incomplete or unclear ascertainment of the causative organism for infections, and competing causes of death (i.e., more individuals may have developed serious bacterial infections had they survived earlier non-bacterial infections).

Profound immune evasion by mirror bacteria could plausibly render humans vulnerable to infection

As discussed throughout Sections 4.1–4.3, it is plausible that the human immune system would be severely impaired if it were to encounter mirror bacteria. Significant uncertainty remains regarding which elements of bacterial immunity would remain functional, and whether these remaining functionalities would be sufficient to stave off mirror bacterial infection. The immune system is extremely complex, with a wide range of microbial sensors and antimicrobial effector mechanisms providing great coverage, diversity, and redundancy that ensure robustness of defense. Furthermore, these different host defense pathways can cooperate, complement, and compensate each other, making it challenging to establish a clear hierarchy of importance during any given infection (Nish & Medzhitov, 2011). Compounding the difficulty of this assessment is the possible diversity in the types

of mirror bacteria that could be created (e.g., Gram-negative or Gram-positive, encapsulated or non-encapsulated), and the natural heterogeneity of the human immune system across individuals. There is also a dearth of experimental evidence and lack of a clear analog for mirror bacteria: they are unlike natural-chirality pathogens in lacking functional virulence factors, yet they are also unlike commensals due to their suspected profound ability to evade immunity. They are also unlike inanimate particles, as they can actively spread and multiply. Simply put, mirror bacteria would represent a unique and unprecedented challenge to the human immune system.

However, several pieces of evidence suggest that the plausible extent of immune evasion exhibited by mirror bacteria could render humans highly susceptible to infection, with potentially harmful outcomes. First, immunodeficiencies affecting even one microbial sensor or effector pathway can already render patients (or animal models) more vulnerable to bacterial infection, as is seen with deficiencies in IRAK-4 or MyD88 (Picard *et al*., 2010), C2 or C4 (Pettigrew *et al*., 2009), or MHC-II (Ouederni *et al*., 2011). Second, individuals who are immunocompromised in a manner that affects multiple cell types or effector pathways—e.g., due to chemotherapy, bone marrow transplants, or immunosuppressive therapy—are often at significant risk of life-threatening infection (Bock *et al*., 2013; Roberts & Fishman, 2021; Safdar *et al*., 2011). Third, profound immune evasion in the form of MAMP masking, phagocytosis inhibition, and antigenic variation, among other strategies, enables various natural-chirality pathogens to block immune clearance, as seen with e.g., *Mycobacterium tuberculosis*, *Salmonella typhi*, *Y. pestis*, and *Neisseria gonorrhoeae*. This can result in long-term, persistent infections that do not resolve until medical intervention (Chandra *et al*., 2022; Demeure *et al*., 2019; Lovett & Duncan, 2018; Monack *et al*., 2004).

These individual pieces of evidence do not make perfect analogies for mirror bacterial infections due to caveats outlined elsewhere in this chapter. However, when taken together, they paint a concerning picture: even if some immune defenses remain functional, mirror bacteria could pose a severe infection risk to humans. In Section 4.4, we discuss what a mirror bacterial invasion could look like if humans were sufficiently exposed to mirror bacteria in the environment.

4.4 Mirror bacteria could plausibly pass barrier surfaces and translocate into the bloodstream and tissues

As described in Sections 4.1–4.3, mirror bacteria would likely evade or impair many elements of the immune system. Consequently, if mirror bacteria were to reach internal tissues or the blood, it is plausible that they would not be efficiently removed by normal immune processes and might instead cause infection (see Section 4.5). Unlike natural-chirality pathogens, however, mirror bacteria would likely not, by default, have functional virulence factors that would facilitate invasion or survival. Furthermore, any virulence factors present could have incompatible chirality with their host ligands.

In this section, we consider whether mirror bacteria would be able to pass barrier surfaces and enter human tissues in the absence of typical virulence factors that normally mediate such processes. As we will discuss, human physical barriers are often leaky due to trauma, environmental compounds, and inflammatory diseases, yielding opportunities for bacteria to translocate. It is difficult to predict how frequently mirror bacteria would bypass these defenses in practice, as that would depend on the

environmental prevalence of mirror bacteria, the efficacy of non-pharmaceutical measures like sterilization techniques or hand-washing, and the robustness of the mirror bacteria to the environmental stressors that limit growth and survival on skin and mucosal surfaces. Nevertheless, commensal bacteria lacking specialized virulence factors for invasion are able to translocate through natural barriers and can be found in the bloodstream (Pat *et al*., 2024), which makes it plausible that mirror bacteria would be able to do the same if present in sufficient numbers and given the opportunity.

While many pathogens invade deeper tissues only after adhesion to—and subsequent colonization of—barrier surfaces like the lungs, skin, or gut, mirror bacteria would plausibly face great difficulty establishing sessile colonies due to a lack of functional adhesion receptors that confer strong adhesion capability. However, adhesion is not an absolute requirement for infection, and comparison with encapsulated bacteria suggests that a non-adhesive phenotype might even predispose mirror bacteria to enter the bloodstream. It is also not clear that absence of adhesion factors would preclude colonization of the lung and other barrier surfaces in all cases, particularly given other unusual properties of mirror bacteria.

Physical barriers are imperfect and can be crossed passively, which could plausibly allow mirror bacteria to invade

Humans encounter environmental bacteria on a continuous basis on body surfaces that are directly exposed to the outside world. These surfaces include the skin, eyes, and mucosa of the respiratory, genitourinary, and gastrointestinal tracts, which are protected by both physical and chemical barriers that keep the vast majority of commensal and pathogenic bacteria from entering the underlying tissues (Marshall *et al*., 2018). Barrier epithelial cells are structurally connected like a fence that selectively allows gasses and nutrients to pass but holds microbes at bay. The structural integrity of the barrier relies on intercellular junctions that connect epithelial cells: protein complexes including tight junctions, adherens junctions, and desmosomes (Matter & Balda, 2003).

Damage to epithelial barriers can allow bacteria to enter the body. Scratches, abrasions, lacerations, burns, and insect bites routinely break the skin and provide portals of entry for microbial infection. Humans also deliberately induce breaches through surgical wounds, needles, and medical and dental implants, and even through less invasive daily actions like flossing and shaving (Brodell & Rosenthal, 2008).

Epithelial damage that is sufficient to allow bacterial translocation can also occur in the absence of trauma. Air pollution-related compounds like smoke and ozone, as well as numerous chemical irritants found in common cleaning agents, have damaging effects on the skin and respiratory tract in case of prolonged exposure, which can result in increased bacterial translocation (Pat *et al*., 2024). Ingested alcohol, fatty acids, and various food additives can disrupt the integrity of the gut epithelial barrier (Portincasa *et al*., 2022), as can many pharmaceutical drugs and medical treatments (Fine *et al*., 2020). Respiratory viral infections disrupt functioning of lung epithelia (Gawrysiak *et al*., 2021; Lalbiaktluangi *et al*., 2023) and can predispose patients to secondary bacterial infections in which bacteria benefit from virus-induced barrier damage to invade deeper underlying tissues

(Lalbiaktluangi *et al*., 2023; Morris *et al*., 2017). Some bacterial pathogens directly cause damage to epithelial cells or tight junctions in order to facilitate invasion (Eichner *et al*., 2017; M. Kim *et al*., 2010). Finally, various non-infectious diseases, such as inflammatory bowel disease, atopic dermatitis, asthma, obesity, and non-alcoholic fatty liver disease, are associated with epithelial barrier dysfunction (Hellings & Steelant, 2020; B. E. Kim & Leung, 2018; König *et al*., 2016; Portincasa *et al*., 2022). Disruptions to epithelial layers allow the translocation of (non-invasive) commensal microbiota (Pat *et al*., 2024). Therefore, it is plausible that mirror bacteria could be similarly translocated as a consequence of such disruptions.

Even in the absence of epithelial or endothelial damage, bacteria can pass physical barriers through natural antigen sampling processes. M cells—specialized epithelial cells found in parts of the gastrointestinal tract and in certain other mucosal-associated lymphoid tissues—have been shown in various mammals to transport particulates like resin and latex beads (Gebert *et al*., 1996; Payne *et al*., 1960) and several bacterial pathogens, including *Salmonella typhimurium*, *Yersinia enterocolitica*, and *Vibrio cholerae*, across the epithelial barrier through both receptor-mediated and endocytic pathways (Ribet & Cossart, 2015). While many of these bacterial pathogens have specialized mechanisms by which to target M cells—mechanisms which would not work even if present in mirror bacteria due to incompatible chirality—given that inert particles can be transported by M cells, it appears at least plausible that mirror bacteria could be transferred across epithelial barriers by M cells. Mouse studies have shown that interstitial dendritic cells also sample commensal bacteria from the gut lumen using transepithelial dendrites and subsequently traffic internalized bacteria to mesenteric lymph nodes (Niess *et al*., 2005). To our knowledge, the presence of antigen-sampling dendritic cells has not been demonstrated in humans, making it unclear whether mirror bacteria could benefit from this or any additional antigen-sampling mechanisms.

Many chemical mechanisms of epithelial barriers would likely be impaired against mirror bacteria

In addition to the physical protection afforded by the epithelium itself, epithelial barriers are protected by a number of chemical mechanisms that mirror bacteria would need to overcome to establish infection. Membranes of the gastrointestinal, genitourinary, and respiratory tracts are protected by a layer of mucus. This layer is primarily composed of glycoproteins called mucins, which form a slimy network with two protective functions. The top layers act like a physical barrier that can bind and trap pathogenic bacteria, whereas the layers closer to the epithelial cells sequester high concentrations of protective AMPs, proteases, and IgA antibodies (Sheng & Hasnain, 2022). Mirror bacteria are expected to have decreased susceptibility to various chemical defenses, which could plausibly allow them to stay close enough to physical barriers to increase the basal translocation rate and aid with potential colonization.

Mucus hinders bacterial invasion through steric hindrance—i.e., providing a physical block. It also adheres to bacteria through both nonspecific mechanisms, such as electrostatic and hydrophobic interactions with bacterial pili and fimbriae, and specific interactions between glycosaminoglycan mucins and bacterial adhesin receptors (McGuckin *et al*., 2011). Steric hindrance and the non-specific interactions are likely insensitive to chirality, but mirror adhesins would not be able to

tightly bind their natural-chirality mucin ligands, plausibly reducing the efficacy of mucus at trapping mirror bacteria. Even so, migration of mirror bacteria in the mucus layer may still be constrained because mirror versions of the mucin-degrading proteases that facilitate movement of natural bacteria are unlikely to degrade natural mucins. On the other hand, even without such proteases, bacteria capable of flagella-based motility can push through the mucin network (Guerry, 2007; Ottemann & Lowenthal, 2002). As such, flagellated mirror bacteria may be able to move through these mucin networks with reduced opposition due to weaker binding forces.

A bacterium that penetrates the top layers of the mucus network will encounter the antimicrobial defenses present in the bottom layer, such as IgA, α-defensins, lysozymes, lactoperoxidases, siderophore inhibitors, and many others (Ribet & Cossart, 2015). AMPs and proteases produced by keratinocytes also comprise the primary chemical defenses of the human skin (Gallo & Hooper, 2012). As discussed in Section 4.2, mirror bacteria would likely be less sensitive to lysozyme and many other antimicrobial enzymes that would be unable to cleave their respective mirror ligands. This could increase the likelihood of mirror bacterial infection because natural-chirality gastrointestinal bacteria like *H. pylori* and *Enterococcus faecalis* that are resistant to lysozyme are more capable of establishing infections in mouse models (Benachour *et al*., 2012; G. Wang *et al*., 2010). Siderophore inhibitors such as lipocalin-2 would likely remain effective, however, as their binding to diverse siderophores is not very stereoselective (Goetz *et al*., 2002) and has been shown to bind both mirror-image and natural-chirality enterobactin (the common siderophore of *E. coli*) with similar affinity (Abergel *et al*., 2006).

It is possible that mirror-specific IgA antibodies would be present at mucosal surfaces (see Section 4.3). IgA deficiency moderately predisposes patients to recurrent respiratory and gastrointestinal infections, suggesting IgA does confer protection against infection. However, this phenotype might partially be due to general disruptions of the microbiome (Swain *et al*., 2019), and it is difficult to determine how mirror bacteria specifically would be affected by the absence or presence of IgA binding.

AMPs that are constitutively expressed at barrier defenses may have some efficacy against mirror bacteria because, as mentioned earlier, many are expected to retain their bactericidal properties. In the intestine, for example, some AMPs are expressed and secreted by intestinal epithelial cells during homeostasis as a means to control the microbiota (Muniz *et al*., 2012; Ostaff *et al*., 2013). Breaches in the mucosal barrier typically result in PRR-mediated increases in the concentrations of these and other, more tightly regulated AMPs (Muniz *et al*., 2012; Ostaff *et al*., 2013). As discussed in Section 4.2, the absence of inflammatory signaling during a mirror bacteria infection might be insufficient to induce additional AMP production. However, the presence of natural commensals alongside mirror bacteria may still cause localized inflammation and, in some cases, might therefore stimulate AMP production and other defenses.

Overall, mirror bacteria would plausibly be more resistant to chemical barriers than natural bacteria. IgA antibodies, AMPs, antimicrobial enzymes, and the physical properties of the mucus layer itself would likely be less effective against mirror bacteria than natural bacteria. The magnitude and significance of this advantage, however, is difficult to determine, a challenge further compounded by the influence of other microbiota on localized inflammatory signaling.

Evidence from transient commensal bacteremias suggests translocated mirror bacteria could end up in the bloodstream

Bacteria that pass the epithelial barriers reach the interstitial spaces, which house tissue-resident immune cells, non-immune cells, extracellular matrix, lymphatic vessels, and blood capillaries. These tissue-resident immune cells typically induce a local inflammatory response upon detection of translocated bacteria, which results in clearance of most, if not all, of these invaders (Walker, 2017). Bacteria that are not locally cleared can be taken up by the lymphatic system and subsequently killed in draining lymph nodes (Siggins & Sriskandan, 2021). Given the immunological impairments described in Sections 4.1–4.3, we expect significantly reduced immunological clearance of mirror bacteria in the interstitium and lymph nodes, potentially creating opportunities for mirror bacteria to disseminate through the body and enter the bloodstream if they were to pass through a breach in the epithelial barrier.

Various human studies demonstrate that bacteria of the indigenous microbiota translocate into deep tissues and the bloodstream in healthy humans on a regular basis without the use of specialized invasion factors. For example, culture-based studies of blood collected from subjects after they undertook "daily oral activities" show that the prevalence of transient bacteremia can be as high as 24% after brushing, 40% after flossing, and 17% after chewing on hard candy (Cobe, 1954; Tomás *et al*., 2012). Furthermore, metagenomic sequencing of whole blood and cell-free DNA in blood from healthy subjects strongly suggests routine bacterial translocation in healthy adults. In the most compelling of these studies, metagenomic analysis of whole blood from 9 770 healthy individuals found that 11% of adults had credible evidence of DNA from presumptive translocating commensal bacterial species (Tan *et al*., 2023). Finally, upward of a quarter of bacterial bloodstream infections lack a known source or focus of infection (Renaud *et al*., 2001; Weinstein *et al*., 1997), suggesting that transient translocation is a possible cause of a significant proportion of bacteremias.

There is additional evidence to suggest that human lymph nodes can be a reservoir for some bacteria, and that bacteria disseminate to the bloodstream through the lymphatic system (Siggins & Sriskandan, 2021). Culture-based studies of patients who undergo a laparotomy for a wide variety of surgical indications showed that live bacteria could be recovered from mesenteric lymph nodes in 5–10% of patients at the time of initial entry into the abdominal cavity (Deitch, 2002; S. Jin *et al*., 2022; Sedman *et al*., 1994). Live bacteria have also been recovered from pelvic lymph nodes of patients with gynecologic tumors (Wells *et al*., 1990) and from cervical lymph nodes in patients with oral cancer (Sakamoto *et al*., 1999). In a study of 1 762 lymph node biopsies from patients with suspected infectious lymphadenitis, bacterial DNA from a variety of species was detected in 30% of biopsies through real-time PCR; when 351 lymph nodes were further cultured on agar plates, 22% of samples yielded live bacteria (Prudent *et al*., 2018).

Another line of evidence suggesting mirror bacteria could passively translocate into the body comes from the observation that inert microplastics of similar size to bacteria have been detected in various human body compartments, including the blood and the heart (Guan *et al*., 2023; Leslie *et al*., 2022; Marfella *et al*., 2024). Although the exact mechanisms of how microplastics enter the bloodstream

are unknown, their initial entry point is thought to be through the epithelial layers of the lungs and the gut (Khan & Jia, 2023).

Because these studies are only snapshots in time, they do not establish how frequently bacterial translocations to the bloodstream occur, although the >5% frequencies across studies suggest yearly incidence is high. The number of bacteria typically translocated in these events, and whether some species have a higher propensity for translocation than others, is unknown. However, given the unusual degree of immune evasion intrinsic to mirror bacteria, it is plausible that mirror bacteria that have passively translocated across epithelial barriers could escape immune clearance and eventually find their way to the bloodstream.

By default, mirror bacteria would likely be weak adherents

Bacterial adhesion to host barrier surfaces is a very common first step in bacterial colonization and invasion for commensals and pathogens alike. Adhesion provides resistance to mechanical removal and facilitates manipulation of host cells through effector systems and toxins (Stones & Krachler, 2016). Additionally, bacterial adhesion to host cells or other surfaces is the first event in the formation of biofilms (Achinas *et al*., 2019). These structured communities of bacterial cells provide protection for their inhabitants from environmental stressors like bacteriophages, immune cells, antibiotics, antibodies, and surfactants (Dunne, 2002).

Adhesion and biofilm-forming capacity vary considerably between individual bacterial species (Malhotra *et al*., 2019; Tu *et al*., 2022) and even between different strains of the same bacterial species, which often differ from each other in the expression of key regulators (Abdulkadieva *et al*., 2022; Martínez-Meléndez *et al*., 2022; Y. Yang *et al*., 2024). As such, the extent to which mirror bacteria would be able to form sessile colonies or biofilms within the human body will highly depend on the specific properties of the mirror bacterial strain. Specialized adhesion mechanisms that confer tight binding are generally stereospecific, and as a result, mirrored versions of natural bacteria would probably not adhere well to specific sites unless they were intentionally engineered to do so.

For concreteness, consider a mirror *E. coli*, which is a plausible model for the first robust mirror bacteria. *E. coli* can form biofilms on abiotic surfaces (Beloin *et al*., 2008), so a mirror *E. coli* could be capable of biofilm formation in some environments. In these cases, adhesion is mediated by non-specific physical-chemical interactions between bacterial appendages and host surfaces that are relatively weak (Dufrêne, 2015; Kreve & Dos Reis, 2021). *E. coli* generally employs a host of different adhesins that confer specific binding. The α-D-mannose-binding fimbrial adhesin FimH seems particularly important for biofilm formation within the human body, where mucosal surfaces are constantly washed by fluids. Point mutations in FimH prevent strong stationary attachment under fluid shear force, so that mutated bacteria instead roll across the surface (B. N. Anderson *et al*., 2007). Because mirror FimH would not be able to bind α-D-mannose (Firon *et al*., 1983; Gade *et al*., 2017), it is likely that an unmodified, generalist mirror *E. coli* would have reduced adhesion and biofilm-forming capacity compared to natural commensal and pathogenic *E. coli* strains.

While a mirror *E. coli* would likely adhere poorly to bodily surfaces, there is no fundamental reason why mirror bacteria could not evolve or be engineered to be better adherents. As discussed in Section

8.4, mirror bacteria could evolve rapidly. A mirror *E. coli* would already possess nonspecific adhesion mechanisms and be capable of biofilm formation, and evolutionary experiments have shown that adhesion and biofilm formation on abiotic surfaces can improve upon positive natural selection (M. Yoshida *et al*., 2022). If improved adhesion would facilitate vertebrate colonization and infection—say, by reducing the exposure to mirror bacteria needed to cause infection—then it could plausibly evolve very rapidly.

Weak adhesion may impair colonization of barrier surfaces, but not preclude systemic infection

Since the gut, urinary tract, and other internal barrier surfaces are subject to aggressive mechanical clearing mechanisms, a weakly adhesive mirror *E. coli* might have difficulty colonizing these areas. Biofilms also protect bacteria from dry, acidic environments, so any impediments in biofilm formation could make skin colonization challenging.

While a reduction in adhesive capacity might hinder colonization of barrier surfaces, it is not a requirement for translocation or infection. Indeed, a weakly adhesive phenotype can confer certain benefits such as improved nutrient access and increased proliferation (Achinas *et al*., 2019), as well as reduced hindrance from the extracellular matrix during translocation (McGuckin *et al*., 2011). FimH-mutated *E. coli* strains that rolled over surfaces under shear stress ultimately colonized larger surface areas than non-mutated *E. coli* (B. N. Anderson *et al*., 2007). Furthermore, a major function of biofilms is to protect bacteria from immune cells, antibodies, antibiotics, and bacteriophages. Since mirror bacteria would already be resistant to most such agents, the need for biofilms as a protective measure would be plausibly diminished.

An analogy for the clinical consequences of a weakly adhesive phenotype can be seen in infections with encapsulated bacteria, which produce exopolysaccharides that increase electrostatic repulsion to the extracellular matrix, mask adhesion molecules, and inhibit phagocytic clearance (Absolom, 1988; Hollands *et al*., 2010; Schembri *et al*., 2004). Encapsulated or mucoid bacterial strains are inherently less tissue adhesive (but more immune evasive), and are more commonly associated with bloodstream infections than with localized biofilm infections (Moxon & Kroll, 1990; Siggins *et al*., 2020). For example, non-encapsulated strains of *Haemophilus influenzae* are more commonly found as colonies in the nasopharyngeal carriage than encapsulated strains; however, the encapsulated strains are strongly linked to invasive disease and bacteremia (Crowe, 1987; Moxon & Kroll, 1990; Oliver *et al*., 2023). Similarly, in a study with 1 100 patients with *Streptococcus pyogenes* infection, encapsulated strains were strongly associated with invasive disease syndromes such as bacteremia, toxic shock syndrome, and rheumatoid fever, but very weakly associated with non-invasive disease like pharyngitis (Johnson *et al*., 1992).

A weakly adhesive or planktonic mirror *E. coli* (or similar generalist mirror bacterium) might be able to colonize niches in the human body so long as there are sufficient nutrients to sustain their proliferation at a rate that outpaces mechanical or immunological clearance (Stones & Krachler, 2016). Colonization of the lungs would be particularly concerning. *Yersinia pseudotuberculosis* can colonize the lungs of mice in the absence of specialized adhesion factors (Paczosa *et al*., 2014), and

P. aeruginosa can establish acute lung infections in planktonic (free-living, non-biofilm) form in human patients (Valentini *et al*., 2018). As such, mirror bacteria might be able to colonize the lungs even without functioning adhesins. The gut would be another concerning site for long-term colonization, but the role of bacterial adhesion in gut colonization is less clear (Lin *et al*., 2024). Whether initial colonization would result in long-term, persistent colonies would likely depend on a wide range of factors, including the ability to access and process available nutrients. Mirror bacteria would have to compete with native microbiota for nutrient access at these sites, but their resistance to many bodily defenses, phages, and most forms of bacterial warfare (see Section 8.1 for a more detailed discussion) could provide significant advantages that might, in some cases, be enough to compensate for reduced access to nutrients and other disadvantages that would result from reversed chirality.

The potential clinical consequences of such an infection are hard to predict, but if mirror bacteria were able to successfully establish a persistent population within some bodily niche, this could provide them with a place to proliferate and disseminate from, increasing the chances of eventual translocation into the bloodstream. It is also possible that successful colonization could cause adverse clinical pathology directly—for instance, by causing gut dysbiosis or pulmonary disease. A more detailed assessment of the potential consequences of mirror bacterial colonization of luminal or exterior spaces would be necessarily speculative, and is beyond the scope of this report.

4.5 Mirror bacteria could plausibly replicate in blood and cause lethal systemic infection

Infection with natural-chirality bacteria spans a vast range of clinical manifestations. The course of infectious disease pathology is very challenging to infer from pathophysiological first principles due to the inherent complexities and heterogeneity of host-pathogen interactions. Assessing general patterns for the clinical course of any mirror bacterial infection would be highly uncertain and speculative. Nonetheless, given the extent to which mirror bacteria may evade immune defenses (Section 4.1–4.3), it appears plausible that if mirror bacteria were to access internal compartments like the blood, this could result in a systemic infection. When uncontrolled, systemic infections from natural bacteria are extremely dangerous, regardless of the properties or severity of any localized infections caused by the same species. The possibility that exposure to mirror bacteria could lead to similar systemic infections is therefore concerning and warrants further assessment.

As discussed in Section 4.4, commensal bacteria frequently translocate across barrier surfaces. Human blood has suitable abiotic conditions and sufficient nutrients to permit the replication of many commensals and other common bacteria. However, bacteria within blood must also face immunological clearance mechanisms, including the complement system, antibodies, and professional phagocytes (particularly within the spleen and liver). So long as these mechanisms remove bacteria more rapidly than they can replicate, any translocated bacteria will be removed before they can establish a persistent bacteremia. Thus, bacteremias associated with passive translocation are typically transient, subclinical, and self-limiting.

Many immunological responses to natural bacteria appear unlikely to function effectively against mirror bacteria (Section 4.1–4.3). In addition, as discussed below, conditions within the blood could be growth-permissive for many mirror bacteria, including mirror strains of generalists such as *E. coli*. It is therefore plausible that mirror bacteria within the bloodstream could replicate without being checked by normal immunological responses. Absent medical intervention, the result would likely be fatal, whether due to a delayed sepsis-like response or other changes to the blood.

Generalist mirror bacteria could plausibly grow in human blood

The blood is a broadly temperate environment for bacterial growth. With a temperature of roughly 37℃, a pH of 7.4, an oxygen tension of 75–100 mmHg, and a water activity of 0.99, abiotic conditions within blood would be growth-permissive for many common bacteria. This is supported by the fact that many bacterial species can proliferate in blood: specialist pathogens, commensals of skin and gut, and environmental bacteria have all caused transfusion-transmitted bacterial infections (Brecher & Hay, 2005).

A mirror bacterium would likely be at a nutritional disadvantage relative to natural-chirality bacteria. Nevertheless, nutrient availability does not appear to be limiting for a sufficiently robust mirror bacterium. Human fasting blood glucose is between 3.9 and 5.6 mM (Wilkinson *et al*., 2024), while growth in *E. coli* strains has been demonstrated at glucose concentrations as low as 0.1 μM (Füchslin *et al*., 2012; Shehata & Marr, 1971). If engineered to catabolize natural-chirality glucose, a mirror *E. coli* or similar generalist bacterium would have an ample carbon source. As discussed in Chapter 1, a number of other nutrients could also be used as a sole carbon source by a mirror *E. coli* K-12 even absent D-glucose catabolism, many of which are present in blood. These include glycerol, L-alanine, and L-lactate, which are all found at concentrations of a few hundred micromolar, and acetate, acetoacetate, pyruvate, and succinate, present in the tens of micromolar (Psychogios *et al*., 2011). A mirror *E. coli* could likely already utilize, or quickly adapt to utilize, most other L-amino acids (Table 1.2).

Another nutritional challenge would be access to trace metals essential for growth. The most important (and best characterized) of these is iron, although the picture for copper, zinc, and manganese appears broadly similar (Coverdale *et al*., 2019; Davidsson *et al*., 1989; Kirsipuu *et al*., 2020). Iron is poorly soluble, and free iron is particularly scarce in blood due to dedicated iron-binding proteins such as transferrin (G. J. Anderson & Frazer, 2017; Raymond *et al*., 2003). Bacteria can scavenge iron in many environments by producing siderophores, small organic compounds that chelate free iron with extremely high affinity and are subsequently reabsorbed by the bacteria. These remain effective in blood as they have a higher affinity for iron than host iron-binding proteins (Miethke & Marahiel, 2007; Wilson *et al*., 2016). Lipocalin-2, a human acute phase protein, targets these siderophores to restrict pathogen access to iron (Flo *et al*., 2004). Specialized pathogens can have further adaptations to counteract this in turn, including producing siderophores that evade lipocalin-2 (Fischbach *et al*., 2006) or possessing alternative means of iron acquisition, including scavenging host heme (Contreras *et al*., 2014).

The reversed chirality of mirror bacteria would likely impair their iron acquisition, although this impairment is of uncertain severity and unlikely to apply universally. Mirror-image and natural-chirality siderophores should chelate ferric iron equally well, although the diminished ability to form biofilms would likely deprive mirror bacteria of the benefits of cooperative production between individual mirror bacteria. Unlike many other immune mechanisms, the broad electrostatic/cation-π binding of lipocalin-2 to a wide variety of siderophores (Clifton *et al*., 2019) appears to be broadly non-stereospecific, and similar binding of lipocalin-2 to natural-chirality and mirror-image enantiomers of one common siderophore, enterobactin, has been experimentally demonstrated (Abergel *et al*., 2006). However, lipocalin-2 production is induced in infection by recognition of MAMPs by TLRs. Since most TLRs are unlikely to recognize mirror MAMPs, it is uncertain whether constitutive lipocalin-2 expression would be sufficient during a mirror bacterial infection to inhibit growth. The alternative mechanisms of iron acquisition (e.g., scavenging heme) have chiral targets, thus their mirror images are unlikely to remain effective.

High concentrations of certain D-amino acids can be toxic to natural-chirality bacteria, as discussed in Box 1.2. Most D-amino acid toxicities require concentrations much greater than found in blood, but D-cysteine is an exception that can cause growth inhibition of *E. coli* at micromolar concentrations in some environments (Soutourina *et al*., 2000). L-cysteine and L-cystine concentrations within serum total around 200 uM (Psychogios *et al*., 2011), and though in blood the majority of cysteine is likely bound to proteins through disulfide bonds (Chawla *et al*., 1984), it is possible that L-cysteine could slow or even entirely preclude growth of an unadapted mirror *E. coli*. However, even if this were the case, it is plausible that resistance to cysteine would arise rapidly (see Box 1.2). The extent to which other mirror bacterial strains would be sensitive to L-cysteine is unclear; *P. aeruginosa*, for instance, shows no inhibition by D-cysteine even at millimolar concentrations when grown in minimal medium. It is conceivable that other compounds in blood could prove toxic to a mirror *E. coli* or other mirror bacteria due to their reversed chirality, but this appears unlikely given both the low concentrations of most metabolites and the direct evidence that, for many metabolites, their enantiomer is not toxic to natural-chirality *E. coli*.

Absent an effective immune response, mirror bacteria could replicate unchecked within the bloodstream

The typical fate of bacteria within the blood is clearance rather than proliferation, as natural pathogens are usually rapidly removed by the immune system (Levin & Antia, 2001). However, mirror bacteria would likely evade many of the immunological defenses that clear natural bacteria from the blood. As discussed in Sections 4.1–4.3, impaired innate immune recognition would considerably reduce the efficacy of professional phagocytes, the complement system, anti-microbial enzymes and peptides, and antibodies. If mirror bacteria are able to replicate faster than they are removed by any remaining immunological defenses, their population within the blood will grow exponentially.

It is plausible that some clearance processes would retain at least partial efficacy, such as the alternative activation pathway of the complement system against Gram-negative bacteria. If these processes are able to remove the mirror bacteria faster than they replicate, this may be protective

against some kinds of mirror bacteria, particularly those suffering from nutritional or other major defects that limit their growth rate. However, given that a wide variety of species can cause transfusion-transmitted bacterial infections (Brecher & Hay, 2005), and that people with relatively mild immunodeficiencies or immunosuppression are at much greater risk of systemic infection (Chaudhary *et al*., 2017; Kristinsson *et al*., 2014), it is plausible that sufficiently robust mirror bacteria could establish infection even within healthy adults.

If the replication rate for a mirror bacterial strain within blood is greater than immune clearance, the presence of even a single bacterium within the blood could lead to infection. In practice, any given mirror bacterium that reaches the blood might die before it replicates, either due to immune mechanisms that would still function against mirror bacteria (e.g., complement) or other processes (e.g., a fatal mutation). If more than a handful of mirror bacteria enter the blood, however, the probability of all of them dying before replicating would be lower, and infection would become more likely. Similar behavior is seen in many natural systemic infections, in which all bacteria present may have descended from as few as a single bacterium (Gerlini *et al*., 2014).

Systemic mirror bacterial infection could plausibly be fatal

If able to establish themselves within the blood, mirror bacterial concentrations would likely grow exponentially as long as they have access to sufficient concentrations of appropriate nutrients. In natural infections, the escalating immune response acts as a negative control on bacterial populations, but this is unlikely to function efficiently in a mirror bacterial infection for the reasons outlined in Sections 4.1–4.3. Instead, the mirror bacterial population could plausibly grow until it severely disrupts vital bodily processes, resulting in host death. In typical natural bacterial infections, death is caused by sepsis, which is a severe immunological dysregulation. An analogous severe immune dysregulation appears to be the most plausible cause of death in a mirror bacterial blood infection. Nevertheless, even if a sepsis-like response is avoided, mirror bacteria in high concentrations would likely deplete nutrients, modify blood chemistry, and cause physical disruptions to blood vessels and organs. Each effect is credibly lethal, and the prospect that an uncontrolled mirror bacterial infection in the blood would not have severe health consequences appears remote.

The usual cause of death from natural systemic bacterial infections is sepsis, a life-threatening condition that arises when the host response to infection injures its own tissues and organs (Singer *et al*., 2016). While the immune system is usually able to protect humans from bacterial infections, if it is unable to do so, mounting pathogen load and tissue damage drive an extreme, dysregulated, and self-reinforcing hyperinflammation response (Jarczak *et al*., 2021).

The pathophysiology of sepsis is incompletely understood. In natural infections, the immune system is activated by many pathways, and it is challenging to determine which mechanisms drive sepsis-associated pathology (van der Poll *et al*., 2017). Stimulation of the immune system by MAMPs or DAMPs appears to play an important role in driving the hyperinflammatory state (Delano & Ward, 2016; van der Poll *et al*., 2017; Wiersinga *et al*., 2014), as does failure of regulatory pathways to limit the induced inflammation (Zhang & Ning, 2021). However, no specific MAMP or DAMP signals appear to be strictly necessary for the condition to develop. For example,

inflammation induced by LPS endotoxin can drive sepsis pathophysiology during Gram-negative bacterial infections (Kellum & Ronco, 2023), but LPS is not present in Gram-positive bacteria and therefore cannot play a role in causing sepsis in those types of infections. A variety of similar hyperinflammatory conditions—grouped under several overlapping diagnostic labels such as Systemic Inflammatory Response Syndrome, Cytokine Storm Syndrome, and hypercytokinemia—can occur after widespread tissue injury, such as those resulting from burns, trauma, or ischemia (Chakraborty & Burns, 2024). This suggests that DAMP production and recognition alone may be sufficient to drive life-threatening hyperinflammation, and that DAMPs may play important roles in mediating sepsis during infection (Cicchinelli *et al*., 2024).

Innate immune activation would be expected to be less potent in the context of a mirror bacterial infection. Compared to natural-chirality pathogens, mirror bacteria would not natively produce immunogenic endotoxin nor virulence factors that would directly damage host tissue; as discussed earlier (Sections 4.1–4.3), most immunological mechanisms of recognition and response to mirror bacteria are likely to be substantially impaired.

Nonetheless, we think sepsis-like immune dysregulation would likely be the most plausible ultimate outcome of an uncontrolled mirror bacterial infection. Although immune recognition of mirror bacteria is expected to be seriously compromised, mirror bacteria remain likely to trigger some immune responses. The complement system, for example, could still be activated through the alternative (and possibly classical) pathway, resulting in the release of the anaphylatoxins C3a and C5a. Both promote inflammation and activate professional phagocytes; C5a in particular plays a crucial role in causing many of the life-threatening effects of sepsis (Ward, 2010). Cross-reactivity of PRRs with mirror macromolecules, which would likely be very weak individually, could plausibly also trigger an immune response if mirror bacterial concentrations expand and the number of those interactions multiplies. Tissue damage caused either indirectly by the immune system or directly due to other consequences of mirror bacterial infection (e.g., by local nutrient depletion, phagocyte cell death, or toxic metabolites) could also trigger PRRs through DAMPs, which could nonspecifically activate immune cells and further drive the sepsis-like response. This residual immunological function triggered by DAMPs may not be competent to clear a mirror bacterial infection, yet still be sufficient to trigger sepsis once this infection becomes overwhelming.

This observation is consistent with the clinical experience of infection in immunocompromised patients: despite the hyperinflammatory nature of the condition, these patients are at an increased risk of developing sepsis (Greenberg *et al*., 2018; Kumar *et al*., 2015; Tolsma *et al*., 2014). Sepsis can occur in serious infection across bacterial species, and does not appear to require any specific pathogen factor. Indeed, to the best of our knowledge, overwhelming systemic bacterial infection essentially always results in sepsis, regardless of the patient's immune status or bacterial species, and as noted above, similar hyperinflammatory conditions also arise in response to widespread tissue damage even absent infection. While the exact processes that would initiate a sepsis-like immunological dysfunction during mirror bacterial infection are unclear, it appears plausible that such dysfunction would ultimately be triggered, and the result could resemble sepsis seen in natural bacterial infection.

Sepsis is lethal through multiple distinct and overlapping mechanisms. Generalized inflammation disrupts and damages most organ systems, resulting in progressive multiple organ failure (Gotts & Matthay, 2016) and hemodynamic collapse ("septic shock"). Perhaps paradoxically, late-stage sepsis also causes immunosuppression, which can lead to the acquisition of secondary hospital-acquired infections (Hotchkiss *et al*., 2013). With intensive supportive care and urgent administration of antimicrobial therapy, sepsis mortality is 25–50% (Cecconi *et al*., 2018); without treatment, sepsis is expected to be fatal within hours or days.

Although a sepsis-like disease appears to be the most plausible outcome of mirror bacterial infection, it is difficult to be certain given the expected properties of mirror bacteria. Another possibility is that the combination of immune evasiveness and limited ability to damage host tissue could result in an unprecedented pathophysiology: uncontrolled infection in internal compartments which does not provoke a severe systemic inflammatory response. If proliferation is not interrupted by host death, mirror bacterial populations could grow to much higher concentrations than typically seen in human or animal models of bacterial disease.

Extremely high mirror bacterial titers in blood are unlikely to be survivable, though the exact pathologies that would first manifest are unclear. A large mass of metabolically active mirror bacteria would consume the nutrients and oxygen carried by the blood. If able to catabolize D-glucose, for instance, mirror bacteria would likely grow until the patient died either from hypoglycemia or hypoxemia, depending on whether D-glucose or oxygen was depleted first. Notably, per unit mass, the metabolic rate of rapidly growing *E. coli* is about a thousand times higher than that of a resting human (Milo & Phillips, 2015); since blood is about 10% of body mass, this suggests that death could occur through this mechanism once mirror bacteria reach a few percent of blood by mass (or $\sim 10^{10}$ cells/mL for a typical bacterium). Even absent D-glucose catabolism, growth on achiral nutrients like glycerol or uric acid, or on other accessible nutrients like L-alanine and other L-amino acids, could eventually lead to mirror bacterial populations large enough to cause hypoxemia, among other problems. Waste products from bacterial metabolism could also cause fatal disruptions to host clinical chemistry, such as acidosis secondary to lactic or acetic acid production.

Very high concentrations of mirror bacteria could also cause physical disruptions. Because mirror bacteria would adhere at most very weakly to the blood vessel endothelium and (in most cases) are unlikely to form biofilms, such physical disruptions would be unlikely to occur until mirror bacterial concentrations become very high. At that point, mirror bacteria could thicken blood and disrupt its flow, leading to a hyperviscosity syndrome (Kwaan & Bongu, 1999). The coagulation cascade is finely controlled, so alterations in viscosity, dilution of coagulation proteins, and other gross changes secondary to high bacterial concentrations could predispose patients to hemorrhage, clotting, or both simultaneously. Mirror bacteria may also diffuse from blood and lymphatics into the end-organs they supply, disrupting their tissues and impairing their functions.

It is not inconceivable that a systemic mirror bacterial infection could stabilize if key nutrients are depleted, although this appears unlikely. Significant depletion of oxygen or glucose from the blood is likely to kill the patient. For a mirror bacterium that cannot consume D-glucose, depletion of achiral and other available nutrients within blood would still plausibly be harmful. Even if growth were nutrient-limited, the infection would only be benign if mirror bacterial concentrations were kept

sufficiently low to avoid an adverse immune response. Even if such stabilization occurred, the equilibrium could be brittle over longer time periods, with later physiological disturbances (e.g., age, intercurrent illness, or even long-term harms caused by a stable mirror bacterial population) pushing the host back into the uncontrolled growth regime.

To summarize, it appears plausible that unchecked mirror bacterial replication could result in a severe and dysregulated inflammatory response similar to sepsis. Even if this was avoided, mirror bacterial infection would probably cause severe harm through a number of other mechanisms. For example, low but chronic levels of inflammation, even if they do not eventually amount to hyperinflammation, are associated with a plethora of health issues ranging from cardiovascular disease to autoimmune disorders and cancer (Furman et al., 2019). The human body is complex, and accurately predicting the consequences of a putative mirror bacterial infection is impossible. Nevertheless, if infection is established and effective treatment is not available (as discussed in Chapter 5), the prognosis would likely be bleak.

The unusual properties of mirror bacteria could create significant potential for misuse

As described throughout this chapter, mirror bacteria would have very unusual properties as potential human pathogens. A mirror bacterium would have unprecedented immune evasion capabilities because of incompatibilities between its mirror macromolecules and natural-chirality host defenses. On the other hand, mirror bacteria would, by default, lack specific adhesion mechanisms and other virulence factors. Even if present, these would mostly be dysfunctional, as they rely on chiral interactions with host proteins. For any given mirror bacterium, it is difficult to know whether immune evasion would suffice to overcome other disadvantages. Nevertheless, it appears plausible that sufficiently robust mirror bacteria, such as mirror *E. coli* strains, could prove to be virulent pathogens without any additional engineering.

If robust biocontainment measures are engineered into mirror bacteria, it should be possible to create specific strains that pose minimal risks to human health (see Section 3.3). However, the fact that, absent robust biocontainment, a generalist mirror bacterium might be a novel and highly dangerous pathogen is very concerning from both a biosafety and, particularly, a biosecurity standpoint. Even if the immune system or other barriers provide some level of protection against lethal mirror bacterial infection, invasive mirror bacteria would rapidly evolve (see Section 8.4) and could therefore become increasingly dangerous to humans.

A malicious actor could intentionally seek to increase the harms caused by a mirror bacterium by providing it with abilities that are unlikely to evolve through natural selection. Many methods for increasing virulence might be technically straightforward if mirror bacteria were created and became generally accessible. Describing potential "weaponization" steps in more detail (beyond merely disabling biocontainment measures) is outside the scope of this report. Nevertheless, it appears plausible that even if some biological barriers make mirror bacterial infection by, for example, a mirror *E. coli* much less likely than anticipated here, these deficiencies could be circumvented by a sufficiently skilled malicious actor. With continued progress in biotechnology in general, and mirror

biology more specifically, it would become progressively easier for a malicious actor to bridge the gap between a “safe” mirror bacterium and a highly pathogenic one.

Chapter 5: Medical Countermeasures

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Public health and medicine have a large suite of interventions to manage infectious disease. Many of these ultimately rely on stereospecific interactions, and thus the reverse chirality of mirror bacteria reduces the breadth and efficacy of potential responses. Adapting interventions (or pioneering new ones) that could be effective versus mirror bacteria would likely be challenging.

This chapter focuses on possible pharmaceutical countermeasures to mirror bacterial infection. Non-pharmaceutical interventions, such as PPE, handwashing, and sterilization techniques, are generally insensitive to chirality and could be used to reduce human exposure to mirror bacteria. Akin to natural-chirality pathogens, it appears unlikely these measures alone would be sufficient to guarantee protection. If a sufficiently invasive mirror bacterium were to become common in many environments, a possibility explored in Chapter 8, then these non-pharmaceutical interventions to reduce exposure would carry significant costs and may not be sufficient to reliably prevent infection, though this would depend on abundances in the environment.

Chapter 4 detailed how mirror bacteria could pose serious risks to human health upon exposure and subsequent infection. Pharmaceutical interventions such as antibiotics and vaccines could potentially offer robust means to prevent infection if administered prophylactically or to cure infections once diagnosed. This chapter considers the prospects of developing antibiotics and vaccines against mirror bacteria, and touches upon other potential pharmaceuticals such as antibody or phage therapies that are used in clinical practice to treat bacterial infections. In principle, adapting these modalities to target mirror bacteria appears feasible; whether they could substantially reduce the plausible danger posed by mirror bacteria to humans is very uncertain, for a number of reasons.

First, antimirror pharmaceuticals that are potent *in vitro* may not prove clinically effective *in vivo*. While mirror bacterial infections in otherwise healthy individuals would be importantly different from natural pathogen infections in immunodeficient individuals, the deficits in the immune system's response to mirror bacteria may resemble severe immunocompromise (see Chapter 4 for further discussion). Infection with natural-chirality bacteria in a severely immunocompromised patient is often fatal despite maximal medical intervention, and prophylactic antibiotic coverage and extensive immunization are not sufficient to ensure a normal life expectancy for those with severe

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immunodeficiencies (Hanna & Etzioni, 2014; Picard *et al*., 2010). It is thus uncertain whether pharmaceutical interventions—or a combination of interventions—could reliably treat or prevent life-threatening mirror bacterial infection.

Second, even for natural-chirality pathogens, discovery and development of a clinically effective pharmaceutical is not easy. Doing so rapidly in the midst of an emerging disease outbreak is typically harder still. Mirror bacteria may pose additional challenges for countermeasure development versus a newly emerging natural-chirality pathogen, including the likely necessity for live organisms to be handled in high-containment facilities, the need for mirror-image reagents and chemistry for experimental manipulation, and little relevant pre-existing expertise on these organisms within the scientific community. Scientific progress over the coming decades may lessen these challenges, but it seems likely they will remain formidable.

Third, the successful development of a highly effective medical countermeasure does not guarantee those who need it will receive it. Large-scale manufacture of a newly developed antimirror countermeasure may be difficult, especially if it demands extensive modification of existing manufacturing capacity, or new facilities to be brought online. A large proportion of the world population lives in low-income countries, where preventable deaths from a lack of access to relatively inexpensive medicines are commonplace (Adenowo *et al*., 2015; UNICEF, 2024): access for these populations to antimirror medical countermeasures, which would probably be substantially more expensive, is likely to be limited. The possible combination of environmental prevalence, frequent human exposure, and limited durability of protection to a mirror bacteria could, in a worst-case scenario, make protection of their population difficult even for the wealthiest nations. Mirror bacteria pose further challenges to other aspects of outbreak responses (e.g., diagnostics, sample management) similar to those noted for countermeasure development, but these are beyond the scope of this chapter.

Last, while in this chapter we focus on protecting humans, mirror bacteria could potentially also infect animals and plants, and invade natural ecosystems (see Chapters 6, 7, and 8). Countermeasures to protect agriculture and the natural environment are discussed in Chapters 7 and 8. As the prospects of preventing or remediating ecological damage caused by mirror bacteria appear to be very limited (see Section 8.6), environmentally invasive mirror bacteria could cause severe harms even if humans could themselves be protected from direct infection through medical countermeasures.

5.1 New antimirror compounds could be developed to target mirror bacteria, but most existing antibiotics would not function

Antibiotics are small molecules that target mechanisms of survival and replication that are absent in humans but present in many or all bacteria. They constitute one of the most important and effective methods to combat bacterial infections. Some existing antibiotics could be repurposed to combat mirror bacterial infection, mirror enantiomers of existing antibiotics could be developed (with varying degrees of difficulty), and antibiotics that exploit the reversed chirality of mirror bacteria to be selectively toxic toward them could possibly be developed.

Some existing antibiotics could be repurposed to combat mirror bacterial infection

Most existing antibiotics function by inhibiting chiral enzymes or targeting other specific chiral biomolecules within bacteria (Kohanski *et al*., 2010). The enantiomers of chiral antibiotics are generally not expected to retain bactericidal activity, as they are unlikely to bind tightly to the biomolecules targeted by their enantiomer. This has been confirmed experimentally in a number of cases, including for chloramphenicol (Maxwell & Nickel, 1954), fosfomycin (Hirota *et al*., 1998), and polymyxin B (Slingerland *et al*., 2022), as well as for enantiomers of specific cephalosporins (Cesta, 2021), oxazolidinones (Brickner, 1996; Fortuna *et al*., 2014), penicillins (Cesta, 2021), and tetracyclines (Korst *et al*., 1968; Muxfeldt *et al*., 1979). The fluoroquinolones, built around a core achiral structure, provide a partial exception with enantiomers sometimes having similar activity (Chu *et al*., 1991; Liu *et al*., 2005), though the enantiomer of levofloxacin (sometimes used racemically as ofloxacin) has much lower activity (Morrissey *et al*., 1996).

Just as the enantiomers of chiral antibiotics usually have little or no antibacterial activity, the chiral antibiotics themselves would usually have little or no antibacterial activity against mirror bacteria. As a result, the utility of antibiotics for the treatment of mirror bacterial infections can be informed by their structures. Chiral antibiotics would be expected to lose activity against mirror bacteria, as can be verified by testing their enantiomers against natural-chirality bacteria. Achiral and racemic antibiotics—those consisting of a mixture of both enantiomers—should by contrast remain active. In Table 5.1 we classify the major structural classes of antibiotics included in the WHO Essential Medicines List (World Health Organization, 2023) by whether they are expected to be stereospecific or not and, if so, the likely ease of producing mirror-image versions to combat mirror bacteria.

Chirality would not be not the sole determinant of susceptibility: in the same way that a particular antibiotic may only be potent against some normal-chirality bacterial species, a given mirror bacteria may not be susceptible to all antibiotics, even if they were of the “correct” chirality. Similarly, susceptibility is not the sole determinant of clinical effectiveness: for example, the pharmacokinetic properties of an antibiotic may make it incapable of reaching a particular site of infection at a sufficient concentration, or may preclude prophylactic use. It is therefore uncertain whether an antibiotic that would be expected to retain its potency across various mirror bacterial species would in fact effectively treat infections caused by a particular mirror bacterial strain.

Achiral and racemic antibiotics—which are expected to be as potent against mirror bacteria as they are against natural-chirality bacteria—are therefore promising therapeutic candidates against susceptible mirror bacterial infections, as they could be straightforwardly repurposed with little further development or clinical safety testing. Of the major antibiotic classes, only (fluoro)quinolones, sulfonamides, and trimethoprim derivatives consist mainly of achiral or racemic members, alongside the miscellaneous antibiotics metronidazole and nitrofurantoin. Co-trimoxazole, an inexpensive broad-spectrum formulation that combines trimethoprim and sulfamethoxazole, is widely used, including in developing nations (Church *et al*., 2015), and quinolones are also among the most commonly used antibiotics globally (Browne *et al*., 2021).

Antibiotic Class	Examples on 2023 WHO Essential Medicines List	Target	Activity vs. mirror	References
Achiral or racemic molecules—plausibly usable immediately				
Fluoroquinolones†	Ciprofloxacin	Topoisomerases	Yes	
	Ofloxacin	Topoisomerases	Racemate	Morrissey *et al*., 1996
Sulfonamides	Sulfadiazine, sulfamethoxazole	Dihydropteroate synthase	Yes	
Miscellaneous	Metronidazole	DNA	Yes	
	Nitrofurantoin	DNA	Yes	
	Trimethoprim	Dihydrofolate reductase	Yes	
Chiral molecules, produced synthetically—enantiomers plausibly synthetically accessible, but safety unclear				
Carbapenems	Meropenem	Penicillin-binding proteins	No	
Oxazolidinones	Linezolid	50S ribosomal subunit	No*	Brickner, 1996
Miscellaneous	Chloramphenicol	50S ribosomal subunit	No*	Maxwell & Nickel, 1954
	Fosfomycin	MurA enzyme	No*	Hirota *et al*., 1998
Chiral molecules, produced through biosynthesis—enantiomers unlikely to be easily synthesized				
Aminocyclitol	Amikacin, gentamicin, plazomicin, spectinomycin, streptomycin	30S ribosomal subunit	No	
Cephalosporins	Cefalexin, cefazolin, cefiderocol, cefixime, cefotaxime, ceftazidime, ceftriaxone, ceftolozane, cefuroxime	Penicillin-binding proteins	No*	Cesta, 2021
Lincosamides	Clindamycin	50S ribosomal subunit	No	
Glycopeptides	Vancomycin	Lipid II	No	
Macrolides	Azithromycin, clarithromycin, erythromycin	50S ribosomal subunit	No	
Penicillins	Amoxicillin, ampicillin, benzylpenicillin, cloxacillin, phenoxymethylpenicillin, piperacillin	Penicillin-binding proteins	No*	Cesta, 2021
Polymyxins	Colistin, polymyxin B	Lipid A	No*	Slingerland *et al*., 2022
Tetracyclines	Doxycycline, tetracycline	30S ribosomal subunit	No*	Korst *et al*., 1968; Muxfeldt *et al*., 1979

Table 5.1: Classes of existing antibiotics and characteristics relevant to their applicability for treatment of mirror bacterial infections
All antibiotics in the 2023 WHO Essential Medicines List are listed, excluding antileprosy and antituberculosis medicines, which tend to be very specialized. Biomolecular targets and mechanisms of action are described in (R. Anderson *et al*., 2012; Kohanski *et al*., 2010) unless otherwise noted. Activity against mirror bacteria is inferred from chirality; for those with an asterisk (*), enantiomer compounds from the class have been tested and found to lack antibacterial activity. †The basic fluoroquinolone scaffold is achiral, but some antibiotics in this class are chiral.

Enantiomers of existing antibiotics could be produced, although this appears to be difficult for most classes

The enantiomers of chiral antibiotics may also be effective against mirror bacterial infections, as they should interact with chiral targets within mirror bacteria identically to how existing antibiotics interact with their targets in natural bacteria. However, unlike achiral and racemic antibiotics, the enantiomers of chiral antibiotics may possess a different pharmacokinetic and side-effect profile than their natural-chirality counterparts, and consequently would require testing and separate regulatory authorization (Center for Drug Evaluation and Research, 1992; European Medicines Agency, 1993). However, this process could be accelerated or abbreviated in the context of a major outbreak.

Large-scale production of a mirror-image antibiotic may be relatively feasible for those chiral antibiotics that are produced synthetically (Table 5.1). These include the carbapenems, monobactams, oxazolidinones, and the miscellaneous antibiotics chloramphenicol and fosfomycin (Brewer & Hellinger, 1991; Cao *et al*., 2019; Foti *et al*., 2021; Hahn, 1967; Papp-Wallace *et al*., 2011). Synthesis would require replacing the natural chirality reagents with their enantiomers, but it is plausible that the enantiomers of these antibiotics could be straightforwardly manufactured.

Most classes of existing chiral antibiotics, however, are predominantly produced in whole or in part via biosynthesis, and the biosynthetic steps cannot be easily adapted to generate the enantiomer of their current product (Fedorenko *et al*., 2015). Given the complexity of these antibiotics, the re-engineering of biosynthetic pathways or the execution of total synthetic routes to produce enantiomeric versions of these antibiotics (Table 5.1) on an industrial scale could present major scientific and practical challenges. It is conceivable that strains of mirror bacteria could be cultivated for these biosynthetic tasks, although this would share many of the same scientific and practical challenges and in addition would raise considerable safety and security concerns (see Chapter 3).

Antibiotics with pore-forming or membrane-disrupting activity could prove an exception to the principle that chiral antibiotics would not be effective against mirror bacteria, as their mechanism of action is often not sensitive to the chirality of the lipid membrane (examples of such antibiotics are listed in Table 8.1 of Chapter 8). In many cases, the enantiomers of these antibiotics have even been shown to be effective at killing bacteria, so these antibiotics would be expected to remain effective against mirror bacteria. Not all membrane-disrupting antibiotics would retain efficacy, however, as some bind to chiral targets in the membrane. Daptomycin, for example, binds to the chiral molecule phosphatidylglycerol, and its enantiomer is much less effective at harming bacteria (Moreira & Taylor, 2022).

Given their lack of specificity, membrane-disrupting antibiotics that would retain efficacy against mirror bacteria are often toxic to humans, which can limit applicability. Gramicidin and bacitracin are the only examples that see occasional clinical use, and they are all but exclusively used for topical infections due to their cytotoxicity (Nguyen *et al*., 2024; Pavithrra & Rajasekaran, 2020); salinomycin and other ionophores see use only in veterinary medicine (Zhou *et al*., 2013). It is therefore doubtful that such antibiotics would prove particularly promising drugs to combat mirror bacterial infections.

Novel classes of antimirror antibiotics could be developed to target mirror bacterial infection

Current antibiotic therapies exploit the comparatively minor biochemical differences between prokaryotes and eukaryotes in order to selectively kill microbes while causing few side effects in human patients. Biochemically, all metabolic processes in mirror bacteria will be very different from those in natural-chirality organisms due to the reversed chirality of their enzymes, providing a much wider range of potential antibiotic targets. For example, metabolic pathways such as glycolysis cannot be targeted by antibiotics because they are highly conserved in all living organisms, yet small molecules that selectively inhibit mirror-image glycolytic enzymes would be credible antimirror antibiotic candidates. This abundance of targets could reduce the cost of discovering effective, safe, readily manufactured antimirror therapeutics and prophylactics.

Existing research may be informative. If a compound X is highly toxic to all existing life, but its enantiomer Y is well-tolerated in humans, then Y may be a potent antimirror compound. For example, the enantiomers of the most toxic known nucleoside analog, including but not limited to those with potent anticancer, antiviral, and antibacterial activities (De Jonghe & Herdewijn, 2022; Niu & Tan, 2015), may be promising antimirror compound candidates. Toxicity studies might assess the comparative safety of candidate antimirror compounds and the minimum inhibitory concentrations of their enantiomers against existing bacteria, with promising candidates entering drug development.

On the other hand, achieving *in vitro* efficacy is typically easy for antibiotic discovery campaigns, and toxicity is typically mediated via highly unpredictable off-target interactions (largely driven by physicochemical properties). Developing new antibiotics from discovery to clinical testing and approval takes years, and historically the interval between discovery and clinical introduction has averaged around a decade (Gigante *et al*., 2022; Hutchings *et al*., 2019), though some of this disappointing track record may be due to limited economic incentives for rapid commercial antibiotic development alongside the scientific challenges (M. Anderson *et al*., 2023). It is unclear whether a dedicated antimirror antibiotic development effort could be expected to outperform this historical average during the urgency of a major outbreak.

The clinical efficacy and overall utility of antimirror compounds is unclear

Even if existing antibiotics or novel antimirror compounds could kill mirror bacteria, they would not necessarily be clinically effective. As discussed in Chapter 4, human immunity is anticipated to be impaired versus mirror bacterial challenge, and infection is frequently life-threatening in roughly analogous immunodeficiencies despite available options for antibiotic treatment. For antimirror compounds to be useful therapeutically, infections would need to be diagnosed and treatment begun before irreversible harm had occurred. It is therefore not clear that post-infection antibiotics would be sufficient to cure a mirror bacterial infection, or to mitigate serious health effects even if they could prevent fatality.

If post-infection antimirror treatments are ineffective or insufficiently reliable, the only available strategy would involve the prophylactic use of antimirror compounds (vaccination may be another

means to prevent infection, discussed below). Prophylactic antibiotics are commonly prescribed for the immunosuppressed or immunocompromised (Picard *et al*., 2011; Seddon & Bhagani, 2011; Taplitz *et al*., 2018), although they are not always sufficient to prevent infection depending on the magnitude of the deficiency. Given the important disanalogies between mirror bacterial infections and natural bacterial infections, it is difficult to know whether antimirror compounds taken prophylactically would be effective. Comprehensive prophylaxis would require antimirror compounds that were safe to use indefinitely, ideally with minimal side effects. Comprehensive prophylaxis for all living humans would likely require antimirrors to be produced at a scale exceeding total current worldwide antibiotic production.

Antimicrobial resistance could also evolve in mirror bacteria in a similar fashion to their natural-chirality counterparts. Mirror bacteria are expected to have a comparable capacity for *de novo* mutation, but would by default have limited ability for horizontal gene transfer (see Section 8.4). As such, resistance mechanisms that depend on point mutations in antimirror targets would be much more accessible to mirror bacterial populations than those reliant on a novel protein (e.g., a hypothetical mirror-image β-lactamase). It is also possible a malicious actor could intentionally engineer antimicrobial resistance to counter existing achiral antibiotics, resist specific known antimirrors, or boost overall resilience to antimirror compounds.

5.2 Conjugate vaccines could plausibly be developed against mirror bacteria

In addition to antibiotics, bacterial vaccines have historically played an important role in protection against bacterial infection (Osterloh, 2022). All existing bacterial vaccines appear to elicit immunity against pathogen-specific chiral antigens, and so pre-existing vaccines would be unlikely to provide effective protection against mirror bacteria. Even if the mirror bacteria share an antigen that is targeted by an existing vaccine, immunological memory is unlikely to extend to the mirrored antigen as B cell receptors, antibodies, and T cell receptors are all chiral molecules (see Section 4.3). A new vaccine specific to the mirror bacterial species would need to be developed.

Bacterial vaccines harness a range of different technologies: inactivated, live attenuated, recombinant protein, protein-polysaccharide conjugate, and outer membrane vesicle vaccines have all been licensed. Newer technologies such as plasmid DNA and mRNA are currently being explored for the development of novel bacterial vaccines (Frost *et al*., 2023; Mba *et al*., 2023; Osterloh, 2022). However, just as mirror bacterial infections are unlikely to stimulate a protective immune response due to the expected inability of the human immune system to process mirror bacterial protein antigens, the same is likely to be true of vaccination strategies that rely on immunogens composed solely of mirror protein antigens. As a result, inactivated, live attenuated, recombinant protein, and outer membrane vesicle vaccines would not be expected to prove effective. Nucleic acid vaccines would also not be effective against mirror bacteria because they cannot encode mirror protein antigens.

On the other hand, conjugate vaccines may be a promising approach against mirror bacteria. Conjugate vaccines stimulate an immune response to a normally non-immunogenic antigen by

conjugating an immunogenic carrier to it (Rappuoli *et al*., 2019). Glycoconjugate vaccines—which consist of a carrier protein or peptide conjugated to a polysaccharide—direct antibody responses against polysaccharides that are prevalent on the surface of bacteria, ultimately resulting in bacterial cell death via opsonophagocytosis or complement-mediated lysis (L. L. Lu *et al*., 2018; Romero-Steiner *et al*., 2006). These vaccines have seen success in the prevention of infections by encapsulated bacteria, as conjugate vaccines against *Streptococcus pneumoniae*, *Haemophilus influenzae* type B (Hib), and *Neisseria meningitidis* have dramatically reduced incidences of infection with these pathogens in children over the past two decades (McHugh, 2020; Pollard *et al*., 2009; Wasserman *et al*., 2021). Peptide-protein conjugates use a similar strategy to direct antibody responses to peptides that are poor immunogens. Although no peptide vaccines are in common use, a number are in development for infectious diseases (Eskandari *et al*., 2021; Ou *et al*., 2020) and cancer therapy (Abd-Aziz & Poh, 2022; Malonis *et al*., 2020). They may be particularly promising for vaccines against mirror bacteria as the reversed chirality of mirror bacterial proteins is expected to render them poorly immunogenic (see Section 4.3).

Conjugate vaccines against mirror bacteria could be developed by conjugating a non-mirror carrier protein or peptide to a mirror polysaccharide or peptide. The non-mirror carrier protein would be expected to stimulate a T cell response that could then assist activation of B cells that recognize the conjugated mirror antigen. Such vaccines would be expected to generate antibodies that bind bacteria bearing the mirror antigen, potentially preventing infection (Figure 5.1).

Although promising, the sufficiency of conjugate vaccine-induced immunity for protection against mirror bacterial infections remains uncertain. While existing glycoconjugate vaccines in clinical use are known to induce immunological memory, a memory response may take four or more days to become established upon re-encountering the antigen, which may not be rapid enough to prevent colonization by bacteria with short incubation periods (Pollard *et al*., 2009). In the context of infection by a rapidly invasive mirror bacterium largely unimpeded by a natural innate immune response, the induction of a memory response may be too slow or simply inadequate. Conjugate vaccines are also unlikely to generate a strong $CD8^+$ T cell response, which could impair efficacy against mirror bacteria capable of surviving opsonophagocytosis or otherwise entering and growing inside cells (Osterloh, 2022; Shepherd & McLaren, 2020).

In the context of substantial impairment of native B cell and $CD8^+$ T cell responses, long-term conjugate vaccine-induced protection would largely rely on the maintenance of high serum anti-polysaccharide antibody titers. Glycoconjugate vaccines can (though do not always) induce persistent antibody titers; for example, antibody titers are maintained at protective levels five years after a single dose of a meningococcal glycoconjugate vaccine in adolescents (Quiambao *et al*., 2017). However, given the failure of key components of the immune system outlined in Chapter 4, serum antimirror antibodies alone may not be sufficient to prevent mirror bacterial infection. If mirror conjugate vaccines are achievable, antibody levels needed for protection are expected to be higher than the usual dosage, and frequent re-boosting to maintain protection may be necessary.

Manufacturing challenges may hinder the timely development and distribution of glycoconjugate vaccines against mirror bacteria due to the need for large-scale mirror polysaccharide production. Current manufacturing of glycoconjugate vaccines typically involves the large-scale fermentation of

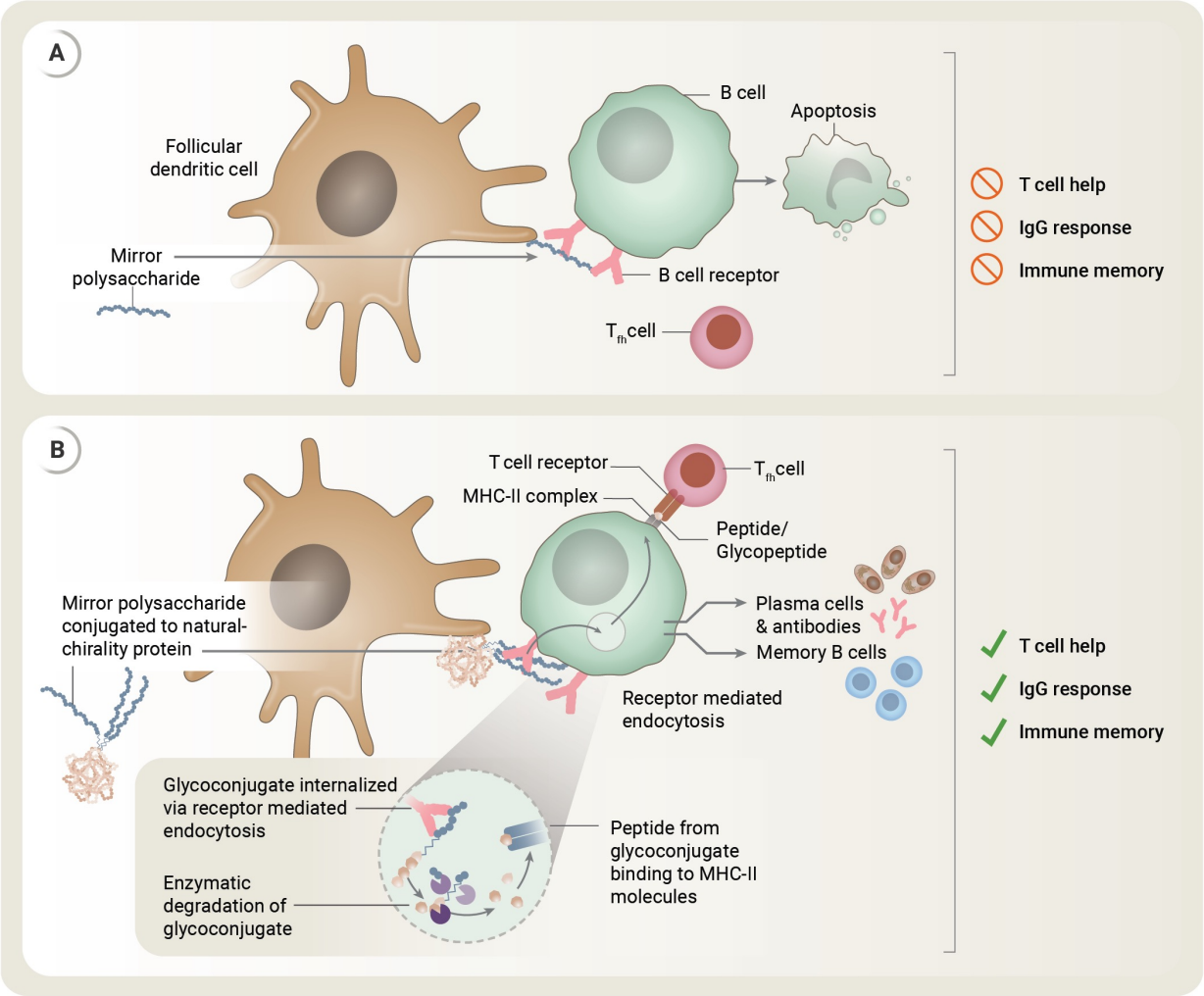

Figure 5.1: Conjugate vaccines may improve adaptive immune recognition of mirror bacteria
A. Expected B cell response against a vaccine composed of mirror bacterial polysaccharides. Follicular dendritic cells and B cell receptors should bind mirror polysaccharides, and their long, repeating structure may activate receptor signaling. However, without the ability to present peptide antigens to and receive signals from T follicular helper (Tfh) cells, these B cells would be unlikely to mount an IgG antibody response or develop immune memory and are likely to undergo apoptosis instead (Section 4.3). **B.** Expected B cell response against a vaccine composed of mirror polysaccharides conjugated to natural-chirality carrier proteins. Upon binding follicular dendritic cells, glycoconjugates are presented to B cells and bind and activate B cell receptors. This results in internalization of the glycoconjugate and proteolysis of the protein carrier into peptides that can be presented to Tfh cells. Peptide presentation results in T cell help and a functional IgG antibody response and immune memory against the target, in this case the mirror polysaccharide. Conjugate vaccines using mirror peptides could also be feasible.

pathogenic bacteria, purification of the desired polysaccharide component, and chemical conjugation to the carrier (Kay *et al*., 2019; Stark *et al*., 2021), although direct synthesis of the polysaccharide component has been successfully used in the Quimi-Hib vaccine licensed in Cuba (Verez-Bencomo *et al*., 2004). Manufacture of antimirror peptide-protein conjugates by chemical synthesis would be more straightforward, though this approach would rely on identifying appropriate peptide targets on the mirror bacterium.

Developing such vaccines as a preparedness measure—that is, in advance of a potential outbreak—would be challenging given the diversity of bacterial antigens a future mirror bacterial species could possess. Developing such a vaccine amidst a mirror bacteria outbreak could be even more challenging, especially if the outbreak is spreading rapidly. Standard timelines for vaccine development span 5–10 years (Pronker *et al*., 2013), although vaccine development and large-scale deployment in a major public health emergency could be much faster, as was seen in the COVID-19 pandemic.

5.3 The efficacy of other countermeasures against mirror bacterial infection is unclear

Although antibiotics and vaccines are the most important medical countermeasures against bacterial infections, there is a mix of rarely used and early-stage therapeutic modalities that could be deployed against mirror bacteria as alternative or adjunct therapies. Compared to antibiotics and vaccines, these other countermeasures tend to have limited (or expensive) production, a much more limited evidence base, or require further technical development, limiting their promise. Other hypothetical countermeasures to mirror bacteria could be enabled by future advances in science and technology, but guessing at their promise and feasibility is beyond the scope of this chapter.

It may be possible to develop antibody therapy against mirror bacteria, but scaling would be difficult

Antibodies can be administered to prevent or treat infection. A broad repertoire of antibodies can be harvested from healthy donors and used as replacement therapy for patients with antibody-related immunodeficiencies (Orange *et al*., 2006). Antibodies with specific activity against a given pathogen can be harvested from humans or animals that have been exposed to the pathogen and form a common element in post-exposure prophylaxis for rabies and tetanus (Forrat *et al*., 1998; Haradanhalli *et al*., 2022). Although more commonly used in non-infectious disease, monoclonal antibodies can be raised to target a specific pathogen target, such as palivizumab for respiratory syncytial virus (Fenton *et al*., 2004).

As a novel pathogen, the chance of any pre-existing antibody therapy being efficacious against mirror bacteria is remote. Most existing monoclonal antibody treatments were developed by the immunization of transgenic mice (R.-M. Lu *et al*., 2020), and given that mirror bacteria are unlikely to provoke an adaptive immune response in vertebrates (see Section 4.3), this strategy would not straightforwardly work. Fully *in vitro* methods such as phage display should, however, remain viable. Even if neutralizing antibodies can be generated, production is expensive (especially if monoclonal antibodies are necessary), and prolonged administration to large vulnerable populations is expected to be infeasible.

Mirror phages could allow for phage therapies to be developed against mirror bacteria

First pioneered in the 19th century, the use of phages to eliminate bacterial infections has seen renewed interest given the mounting challenge of antimicrobial resistance. Besides offering an alternative treatment modality, combination treatments of phages and antimicrobials could have synergistic effects in cases where phage-resistance adaptations in bacteria increase their antimicrobial sensitivity (Hitchcock *et al*., 2023; Lin *et al*., 2017; Oromí-Bosch *et al*., 2023). Except for a few countries, however, phage therapy is largely restricted to experimental or compassionate use (Hitchcock *et al*., 2023; McCallin *et al*., 2019).

Reversed chirality renders mirror bacteria completely resistant to all existing bacteriophages, so mirror phages would need to be created to target mirror bacterial species. Creating such mirror phages is not yet possible, but would likely be easier than creating mirror bacteria; given an outbreak of mirror bacteria it should be possible to create mirror phages by creating mirror-image genomes copied from natural phages. Analogous procedures have already been used to create (natural-chirality) phiX174 and T7 bacteriophage genomes from synthetic oligonucleotides (Chan *et al*., 2005; Smith *et al*., 2003). Numerous synthetic phages have also been generated from cell-free systems, including for bacteria exhibiting transformation efficiencies too low for *in vivo* production (Mitsunaka *et al*., 2022).

Mirror phage therapy may have some advantages compared to non-mirror phage therapy. Mirror phages are likely more resistant to clearance by the immune system, and because the phages would be exclusive to mirror species, there would be no risk of horizontal gene transfer with natural organisms. Mirror phages, however, could also present some additional risks. As noted in Section 8.4, mirror bacteria would by default lack efficient mechanisms for horizontal gene transfer. While *de novo* mutations would accumulate and cause the initial strain to diversify, adaptations could not spread between strains. By providing a mechanism for horizontal gene transfer, mirror phages could potentially accelerate the evolutionary processes.

Even aside from the more unusual challenges associated with a mirror phage, phage therapy remains nascent. The clinical efficacy of a hypothetical “mirror phage therapy”, especially in the context of deficient host responses to mirror bacteria, is unknown. It is also unknown whether mirror bacteria would evolve resistance to clinical mirror phage therapies. There are likely significant practical challenges in designing an effective mirror phage and deploying it at a large scale. The prospects of using mirror phages as a countermeasure to mirror bacteria in the environment are considered further in Chapter 8.

Chapter 6: Animal Infection

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Chapter 4 describes the reasons why a mirror bacterium could be expected to evade many of the immunological defenses found in humans. A sufficiently robust mirror bacterium, if translocated past the body's epithelial barriers, could plausibly cause a life-threatening systemic infection.

In this chapter, we consider whether mirror bacteria could pose similar risks to other animals. Many aspects of the innate immune system are conserved between vertebrates and invertebrates (Buchmann, 2014; Hoffmann *et al*., 1999; Janeway & Medzhitov, 2002), including the use of specific pattern recognition receptors (PRRs) that recognize chiral ligands (Brennan & Gilmore, 2018) and the killing of bacteria through phagocytic cells (Hartenstein, 2006) and antimicrobial peptides (Zasloff, 2002). As is the case in humans, these mechanisms may be relatively ineffective against mirror bacteria. However, given the largely underexplored diversity of animal species (at least immunologically), it is plausible that some animals would be able to mount a robust defense. Variation in susceptibility could also arise due to diversity in physical barriers to bacterial infection, as well as physiological and behavioral differences.

Section 6.1 discusses other vertebrates, which are immunologically similar to humans and possess an adaptive immune system (Flajnik, 2018). The expected defects in the human immune system—which are described in detail in Chapter 4—will likely be shared by most other vertebrates, suggesting that many vertebrates would be similarly susceptible to infection. Transmission of mirror bacteria between infected vertebrate hosts could facilitate their spread through the environment and, ultimately, transmission from the environment to humans.

Section 6.2 discusses invertebrates, focusing on insects and nematodes. In the best studied insect model, the fruit fly *Drosophila melanogaster*, antibacterial immunity primarily relies on recognition of peptidoglycan, which is chiral. Like the mirror microbe-associated molecular patterns (MAMPs) discussed in Chapter 4, mirror peptidoglycan is unlikely to be recognized by insect PRRs. It therefore appears plausible that many insects would also be vulnerable to mirror bacterial infection. Not only are insects ecologically crucial, they also play important roles in disease transmission, including

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between non-human vertebrates and between non-human vertebrates and humans. As a result, insects could provide another potential route for mirror bacteria to spread.

By contrast, the model nematode *Caenorhabditis elegans* is bacterivorous and protects itself by not ingesting pathogenic bacteria and by recognizing specialized pathogen signals within the intestines. Mirror bacteria would likely be recognized as prey but not as pathogens, and may not be killed by normal digestive processes involving lytic enzymes. This might allow mirror bacteria to persist within the intestinal tract and cause potentially fatal infection, though it is also plausible that mirror bacteria would simply be excreted along with other waste. Compared to insects and nematodes, the molecular details of pathogen recognition in other invertebrate immune systems are relatively poorly studied, making it difficult to draw more general conclusions about their susceptibility to mirror bacterial infections.

6.1 Vertebrate susceptibility to mirror bacterial infection would likely be similar to that of humans

All vertebrates (with the partial exception of jawless vertebrates[9]) possess an innate and adaptive immune system closely analogous to that of humans (Flajnik, 2018; Riera-Romo *et al*., 2016). Many of the expected deficits in the human immune response to mirror bacteria described in Chapter 4 should therefore be shared by mammals (Tizard, 2023), birds (Kaspers *et al*., 2021), reptiles (Zimmerman, 2020), amphibians (Ruiz & Robert, 2023), and jawed fish (Rauta *et al*., 2012). Physiological differences among vertebrate species, particularly differences in immune function, would result in variations in susceptibility to mirror cell infection. For example, Atlantic cod have lost the genes essential for processing and presenting antigens via major histocompatibility complex class II (MHC-II) molecules and have expanded repertoires of MHC class I (MHC-I) and Toll-like receptor (TLR) genes (Solbakken *et al*., 2016; Star *et al*., 2011). Similarly, zebrafish have roughly ten times as many nucleotide-binding domain and leucine-rich repeat-containing (NLR) genes as humans or mice (Howe *et al*., 2016). Most of these immune receptors remain uncharacterized, and some may bind achiral MAMPs, allowing at least partial recognition of mirror bacteria in these species. Nevertheless, it appears likely that a mirror bacterium capable of infecting humans could also infect many species of jawed vertebrates.

Most vertebrate immune mechanisms are chirality-specific and thus would likely be defective

The immune systems of jawed vertebrates are highly conserved. Vertebrate innate immunity relies on chiral-specific PRRs, such as TLRs and NLRs, to detect and respond to conserved features of microbial pathogens such as peptidoglycan and lipopolysaccharide (Buchmann 2014; Magnadóttir,

[9] Lamprey and hagfish have a form of adaptive immunity that does not rely on antigen presentation by MHC molecules (Boehm *et al*., 2018). It is unclear whether these alternative adaptive immune systems rely on the processing and presentation of peptide antigens, making it difficult to assess whether they would be sensitive to mirror bacteria. Lamprey and hagfish innate immune systems are expected to be broadly similar to that of other vertebrates.

2006; Neerukonda & Katneni, 2020; Riera-Romo *et al*., 2016). Vertebrates also use the complement system, professional macrophages, and lysozyme to target and kill foreign microorganisms (Riera-Romo *et al*., 2016). Adaptive immunity based on the use of MHC molecules that present pathogen-derived peptides to elicit adaptive cellular and humoral immune responses is conserved across jawed vertebrates (Flajnik, 2018).

As discussed in Chapter 4, each of the aforementioned mechanisms will likely be at least somewhat defective against mirror bacteria in humans. Given that immunological differences between human and other jawed vertebrates are relatively slight, the immunity of jawed vertebrates could be similarly impaired.

A mirror bacterium capable of infecting humans could potentially infect many vertebrates

Like humans, other vertebrates are susceptible to infection by a diverse range of both obligate and opportunistic bacterial pathogens (Prescott *et al*., 2022). Some are specialists, while others are capable of infecting a broader range of hosts (Shaw *et al*., 2020). Common bacterial pathogens, including *Escherichia coli*, *Bacillus anthracis*, *Staphylococcus aureus, Salmonella enterica*, and *Pseudomonas aeruginosa*, are all capable of infecting a broad range of mammalian and avian hosts, while the last two are also capable of infecting some reptiles and amphibians as well as plants (Herczeg *et al*., 2021; Mermin *et al*., 2004; Pasmans *et al*., 2020; Schikora *et al*., 2012). All but *B. anthracis* have been found, at least occasionally, within fish, though only *P. aeruginosa* is known to be pathogenic within fish (Gauthier, 2015; Ziarati *et al*., 2022). Many other bacterial species can function as human pathogens if given the opportunity—a recent literature survey reported 1 513 known bacterial pathogens (Bartlett *et al*., 2022)—and there is little reason to expect other vertebrates to be more resistant to bacterial pathogens than humans. A mirror bacterium capable of evading human immunity may therefore be able to infect many vertebrate species.

Temperature strongly influences the growth rates of bacteria, and physiological variation in vertebrate body temperatures could influence their susceptibility to infection. Birds and mammals, for instance, typically maintain constant body temperatures between 35–42℃ (Clarke & Rothery, 2008), which is optimal for the growth of *E. coli* and many other human pathogens. Reptiles, amphibians, and most species of fish, though, are generally ectothermic. However, most environments are still warm enough to support *E. coli* growth—which has a minimum temperature requirement of about 8℃ (Baka *et al*., 2013; T. Ross *et al*., 2003) with some variation between strains—for at least part of the year, albeit at lower growth rates. Temperatures in the deep ocean, and in the polar waters are around −1℃ (Kirchman *et al*., 2009; Yasuhara & Danovaro, 2016), so ectothermic fish living in these environments would not be susceptible to mirror *E. coli* infection.

Gastric acidity also varies between vertebrates: in mammals and birds, gastric pH varies from a low of 0.7 in the common buzzard (Herpol, 1967) to 6.7 or higher in sloths (Denis *et al*., 1966). With a fasting pH of around 1.5 (Dressman *et al*., 1990; Russell *et al*., 1993), the human stomach is much more acidic than those of most other mammals, but similar to those of many avian species (Beasley *et al*., 2015). This suggests substantial variability in infection risk from the foodborne transmission of mirror bacteria. Given that *E. coli* frequently pass through mammalian stomachs unharmed and can

maintain growth at any pH above 4 in the intestines (Xu *et al*., 2020), a mirror *E. coli* could presumably do the same. Other physiological and behavioral differences among vertebrates, including variation in skin and mucosa anatomy (Akat *et al*., 2022; A. A. Ross *et al*., 2019), dietary habits, and gut microbiota (G. Huang *et al*., 2022), could affect susceptibility to mirror bacterial infection.

Like humans (discussed in Section 4.4), other vertebrates would likely suffer from leaky barrier defenses. Bacterial entry can occur through open wounds and oral microabrasions. Bacterial translocation also occurs in both the lungs and intestines, though outside humans, the only experimental studies of translocation frequency are limited to mice, which exhibit rates similar to those in humans (Berg, 1999; Ohsugi *et al*., 1992; Wells *et al*., 1988). It therefore seems plausible that a mirror bacterium capable of infecting humans would also be capable of infecting many other vertebrates.

Mirror bacterial proliferation during infections could be fatal and may promote environmental spread

In most vertebrates, the consequences of a mirror bacterial infection are likely to be similar to those described for humans in Chapter 4. Systemic bacterial infections are usually rapidly fatal due to sepsis, a severe immunological dysregulation. Because mirror bacteria would evade most immunological recognition and would, by default, lack toxins or other tissue-damaging virulence factors, it seems possible that mirror bacterial populations within a vertebrate host could become large prior to adverse effects on the host. In humans, it nevertheless appears plausible that very high mirror bacterial concentrations would eventually result in potential fatal sepsis-like immunological dysregulation (see Section 4.5). Even if this were somehow avoided, mirror bacteria within the bloodstream could cause lethal harm through other mechanisms, such as by depleting critical nutrients or by modifying the flow and coagulation of blood.

Unlike humans, the cadavers of non-human vertebrates (particularly wild animals) are usually left to decompose in the environment, which could promote the spread of mirror bacteria. After death, the immune system ceases to function, and mirror bacteria would face competition from endogenous gut-associated bacteria and, after corpse rupture occurs, from environmental bacteria as well (Metcalf *et al*., 2013). Changes to the internal environment within the cadaver, including the loss of body heat and rapid depletion of internal oxygen (Carter *et al*., 2007), may also disfavor continued mirror bacterial growth after death. Nevertheless, a large mirror cell population would likely be present in the cadaver for many days after death (the longer-term fate of mirror bacteria within the environment is considered in Chapter 8). Indeed, mirror bacterial numbers upon death might be higher than in typical bacterial infections due to the expected immune deficiencies. This larger mirror bacteria population could increase the length of time that live mirror bacteria persist in the cadaver and, therefore, the overall probability of transmitting mirror bacteria to new hosts. Transmission to new vertebrate hosts might occur either directly through scavenging, or indirectly through insects or other vectors.

Prior to death, an infected vertebrate might transmit mirror bacteria to other vertebrates through a variety of mechanisms including bites and scratches, fecal contamination of food, water, or soil, consumption of infected animals by susceptible predators, and via arthropod vectors such as mosquitoes, ticks, fleas, and lice. Transmission could occur between wild animals, spreading mirror bacteria through the environment (see Section 8.4), from animals to humans (Rahman *et al*., 2020), or from humans to humans. Unlike most zoonotic bacterial infections, which are limited to a subset of animal species, mirror bacteria could exhibit an extremely broad host range, with transmission also possible between livestock and pet species.

6.2 Many invertebrates would likely be susceptible to mirror bacteria

Invertebrates lack adaptive immunity, but many basic features of innate immunity are shared with vertebrates (Pradeu *et al*., 2024). Vulnerabilities analogous to those of the vertebrate innate immune system would therefore likely be present in many invertebrates. Since most invertebrate immune systems are poorly characterized compared to vertebrates, we have focused our assessment on the best-studied examples.

Insect immune systems may fail to recognize mirror bacteria

In Box 6.1, we discuss the immune system of the model organism *D. melanogaster*, the best-studied invertebrate (Buchon *et al*., 2014). Essentially all antibacterial immunity in *D. melanogaster* is downstream of peptidoglycan recognition. As discussed in Chapter 4, mirror versions of chiral MAMPs such as peptidoglycan would likely not be recognized by vertebrate PRRs. For similar reasons, insect PRRs are also unlikely to recognize mirror MAMPs, rendering an effective immune response unlikely (Figure 6.1).

Mosquitoes and bees also rely on peptidoglycan recognition to trigger antibacterial responses (Evans *et al*., 2006; Liu *et al*., 2019; Liu *et al*., 2020; Rodgers *et al*., 2020; S. Wang & Beerntsen, 2015; Q. Wang *et al*., 2019). However, bees and fruit flies are not particularly closely related—both are within the superorder Holometabola, a group that also contains butterflies, ants, and beetles (Misof *et al*., 2014). It is therefore plausible that reliance on peptidoglycan recognition is conserved across many insects, although too little is known to be certain.

Other aspects of immunity that rely on chiral interactions may be similarly defective. Antimicrobial enzymes are expected to have chiral mechanisms of action and are unlikely to retain potency against mirror bacteria. Lysozymes, for example, cleave peptidoglycan and are important components of early innate immune responses in a range of insects, especially against Gram-positive bacteria (Eleftherianos, Zhang *et al*., 2021). This enzymatic activity would be unlikely to function against mirror bacteria because the lysozymes would likely fail to bind to mirror peptidoglycan. However, many insect genomes encode multiple lysozymes (Hillyer, 2016; Van Herreweghe & Michiels, 2012), some of which lack muramidase activity and might function via achiral mechanisms, akin to some antimicrobial peptides (AMPs).

Box 6.1: Mirror bacterial infection would likely be fatal to *D. melanogaster* due to defective peptidoglycan recognition

Antibacterial immunity in *D. melanogaster* is triggered almost exclusively by the presence of peptidoglycan and its breakdown products (Buchon *et al*., 2014). Peptidoglycan, a chiral polymer present in bacterial cell walls (Pazos & Peters, 2019), is recognized by a protein called Gram-negative binding protein (GNBP) and members of the peptidoglycan recognition protein (PGRP) family (Cherry & Buchon, 2022; Liegeois & Ferrandon, 2022). Recognition activates the Toll and Imd signaling pathways, which regulate the production of antimicrobial peptides that constitute the main defense against systemic infection (Cherry & Buchon, 2022; Liegeois & Ferrandon, 2022). Other immune responses to bacteria, including phagocytosis, melanization[10], nodulation, autophagy, and immune priming are also downstream of innate immune sensing (Hillyer, 2016) and would be unlikely to function normally against mirror bacteria.

PGRPs would also be unlikely to bind or be activated by mirror peptidoglycan. Structural studies have revealed binding interfaces between PGRPs and peptidoglycan that span chiral centers (Chang *et al*., 2004; Chang *et al*., 2006; Leone *et al*., 2008; Lim *et al*., 2006; Reiser *et al*., 2004). Although the structural basis of peptidoglycan binding by GNBP is unknown, it might be driven by similar interactions that would be disrupted upon interaction with mirrored peptidoglycan. GNBP appears to process peptidoglycan to enhance detection via PGRPs and does not trigger the antibacterial pathways by itself (Filipe *et al*., 2005; Gobert *et al*., 2003; L. Wang *et al*., 2006). Therefore, the expected inability of mirror peptidoglycan to bind PGRPs suggests that the *D. melanogaster* immune system would be unable to detect and respond to a mirror bacterial infection.

Adult flies deficient in PGRPs or GNBP fail to express antimicrobial peptides when challenged with a range of Gram-positive and Gram-negative bacteria and succumb to bacterial infections at much higher rates than wild-type flies (Bischoff *et al*., 2004; Gobert *et al*., 2003; Gottar *et al*., 2002; Iatsenko *et al*., 2016; Michel *et al*., 2001; Rämet *et al*., 2002; Takehana *et al*., 2004). It is not uncommon for 100% of PGRP or GNBP-deficient flies to die from bacterial infections that most wild-type flies survive, including infections by bacteria such as *E. coli* that are not typically *D. melanogaster* pathogens. Knockout of the downstream Imd signaling pathway also results in susceptibility to lethal *E. coli* infection (Rutschmann *et al*., 2000). Given that otherwise benign infections can kill flies

[10] In mosquitoes and wax moths, melanization reactions of varying intensity can be triggered by the injection of beads composed of dextran or coated with chiral materials, but not glass beads (Barreaux *et al*., 2017; Schwartz & Koella, 2004; Wiesner, 1992). Although these responses might be mediated by a form of immune recognition, the precise mechanisms have not been identified, and it remains unclear whether mirror bacteria would trigger similar responses. We have been unable to locate similar studies in *D. melanogaster.*

unable to detect peptidoglycan, it seems likely that mirror bacteria could be fatal to adult flies.[11]

Unlike other forms of antibacterial immunity in *D. melanogaster*, production of reactive oxygen species (ROS) within the gut is triggered by uracil (Lee *et al*., 2013; Lee *et al*., 2015), which is achiral. While uracil is present in all living cells, it is not usually released by bacteria except under conditions of stress (Rinas *et al*., 1995), and symbionts within the *D. melanogaster* gut do not elicit ROS generation (S.-H. Kim & Lee, 2014). Uracil instead appears to be a specific signature of the catabolism of uridine obtained from the gut by enteric pathogens (E.-K. Kim *et al*., 2020). Since mirror bacteria would not possess the necessary enzymes to catabolize this exogenous, chiral molecule, they would be unlikely to trigger ROS production in this way.

Insects produce a diverse range of AMPs (Vilcinskas, 2013; Wu *et al*., 2018). As in vertebrates (see Section 4.2), many of these AMPs function by disrupting bacterial cell membranes through mechanisms that are insensitive to chirality, and should therefore remain harmful to mirror bacteria. The enantiomers of two cecropins and of melittin, for example, are both bactericidal (Bland *et al*., 2001; Wade *et al*., 1990), which implies that these AMPs should retain efficacy against mirror bacteria. Other AMPs, however, have chiral targets. Enantiomers of apidaecin, droscin, pyrrhocoricin, and thanatin (found in honeybees, *D. melanogaster*, European firebugs, and the spined soldier bug) appear to be inactive (Bulet *et al*., 1996; Casteels & Tempst, 1994; Fehlbaum *et al*., 1996; Kragol *et al*., 2001), while enantiomers of two peptides produced by green bottle flies show markedly reduced activities against bacteria (Hirsch *et al*., 2019). AMPs are often released in response to pathogen recognition, so even those that would retain activity against mirror bacteria may not be produced and deployed at high concentrations, given the anticipated defects in immunological detection.

Hemocytes are analogs of the professional phagocytes in vertebrates (Browne *et al*., 2013) and would be expected to suffer from similar defects to those described in Chapter 4. Hemocytes recognize bacteria directly via transmembrane phagocytic receptors or indirectly via opsonins (Browne *et al*., 2013; Lin *et al*., 2020; Melcarne *et al*., 2019). In *Drosophila*, for example, the phagocytic receptor Draper binds to lipoteichoic acid on *S. aureus* (Hashimoto *et al*., 2009). Although the molecular ligands for most insect phagocytic receptors are not well characterized (Eleftherianos, Heryanto *et al*., 2021), many are likely similar to the vertebrate phagocytic receptors discussed in Chapter 4 and probably have chiral ligands. Recognition of mirror bacteria is therefore likely to be impaired. The processes of particle engulfment, phagosome maturation, and bacterial killing are also broadly similar between insects and vertebrates (Eleftherianos, Heryanto *et al*., 2021); thus, if mirror bacteria were internalized, they would likely resist the killing activity of antibacterial enzymes and

[11] There appear to be no studies of immune responses in PGRP knockout *D. melanogaster* larvae. Larval exposure to bacteria may be even higher than adult fruit flies, given that larvae eat dead and decaying matter.

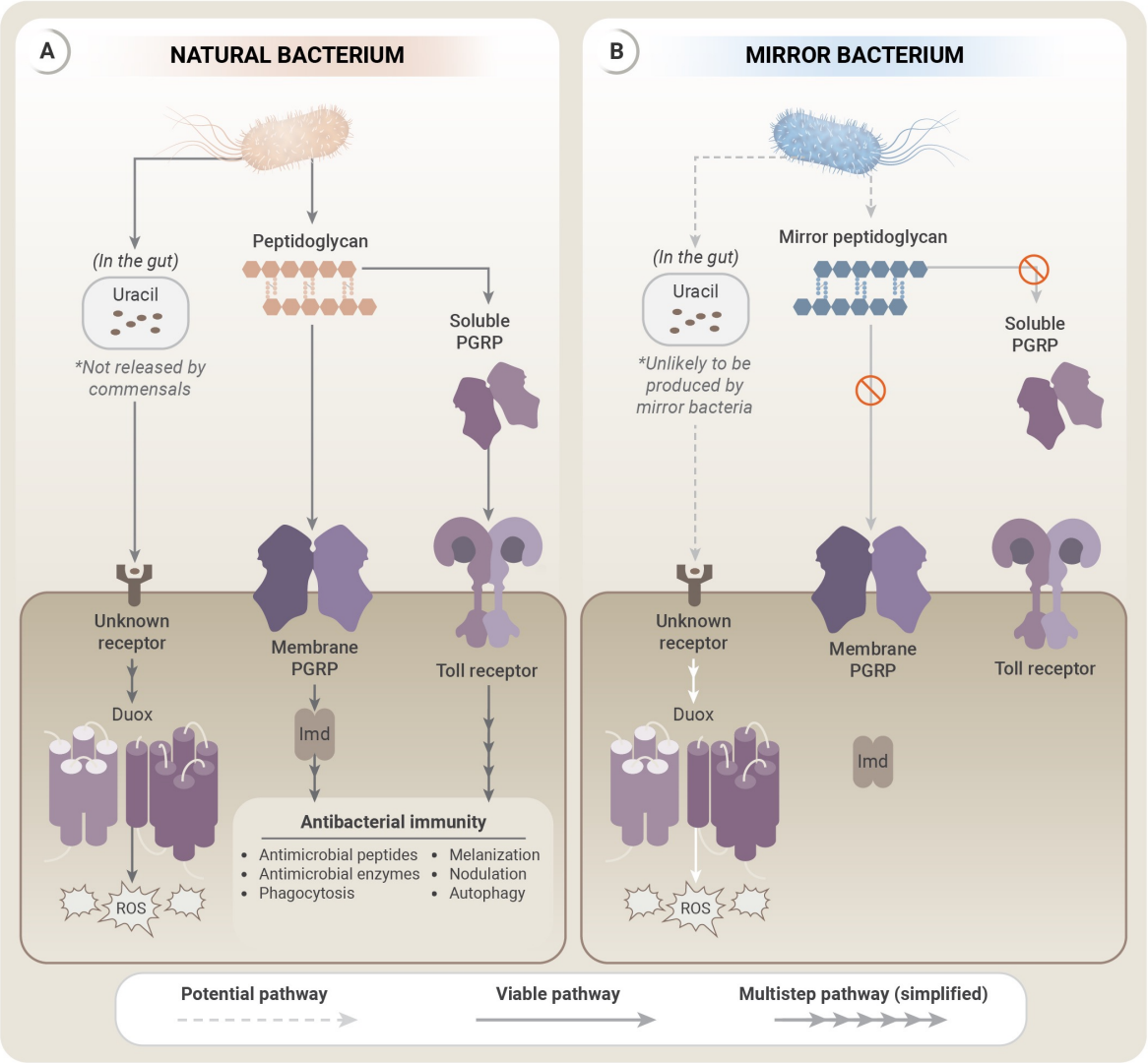

Figure 6.1: Mirror bacteria would likely evade *D. melanogaster* immune mechanisms due to defective peptidoglycan recognition
A. Natural-chirality bacteria trigger innate immunity in *D. melanogaster* primarily through the recognition of peptidoglycan and its breakdown products by multiple innate immune receptors. Membrane-bound peptidoglycan recognition protein (PGRP) receptors bind peptidoglycan and signal through the Imd pathway. Soluble PGRP molecules bind to peptidoglycan, which indirectly activates the Toll pathway via membrane-bound Toll receptors. Both pathways induce antimicrobial peptides, phagocytosis, autophagy, and other mechanisms of anti-bacterial immunity. **B.** Mirror bacteria are unlikely to trigger innate immunity in *D. melanogaster*. Because peptidoglycan is a chiral molecule, PGRPs are unlikely to recognize it, leading to absent or severely impaired antibacterial immune responses. Although uracil, which is achiral, can trigger production of reactive oxygen species (ROS) within the gut (A), it appears to be released only by enteric pathogens when they catabolize exogenous uridine, which is chiral. Mirror bacteria would be unlikely to catabolize by natural-chirality uridine and would therefore be unlikely to produce uracil.

antimicrobial peptides with chiral mechanisms. Even if mirror bacteria were killed by pore-forming peptides or oxidative damage within the phagosome, hemocytes may be unable to digest the remains efficiently.

Mirrored generalist bacteria could plausibly cause lethal infections in insects

Despite the substantial physiological differences between mammals and insects, some bacteria are capable of causing opportunistic infections in both. Both *D. melanogaster* and the moth larvae *Galleria mellonella*, for example, are used as infection models for a wide range of human pathogens (Apidianakis & Rahme, 2009; Tsai *et al*., 2016). In most experiments, high inocula are used to kill the insects because immune defenses are otherwise able to clear the infection. Nevertheless, some strains of *P. aeruginosa* and *Burkholderia* are virulent pathogens in humans as well as *D. melanogaster* and/or *G. mellonella* (Jander *et al*., 2000; Mulcahy *et al*., 2011; Schell *et al*., 2008). Other species that can cause opportunistic infection in both humans and insects include *Bacillus thuringiensis* (Ghelardi *et al*., 2007), *Serratia marcescens* (Hejazi & Falkiner, 1997; Raymann *et al*., 2018), *Enterococcus faecalis* (Cabrera *et al*., 2023), and the plant-pathogen *Agrobacterium tumefaciens* (Gottar *et al*., 2002; Southern, 1996). The ease with which many bacterial pathogens can cause infection in many different hosts, if given the opportunity, suggests that a sufficiently robust mirror bacterium might likewise be capable of infecting a broad range of hosts. While the infection of immunocompromised animals by natural-chirality bacteria is not a perfect analogy for a mirror bacterial infection, the fact that both immunocompromised *D. melanogaster* (see Box 6.1) and immunocompromised humans (Bartlett *et al*., 2022) are susceptible to a broad and overlapping set of opportunistic pathogens is concerning.

To illustrate this more concretely, consider *E. coli*, which is not only a mammalian commensal but also the most commonly studied bacterial species in microbiology and biotechnology. While not traditionally considered an insect pathogen, *E. coli* can survive and replicate within insect hosts (Ciesielczuk *et al*., 2015; Kobayashi *et al*., 1999; Koga *et al*., 2022; Rochon *et al*., 2004; Van den Bergh, 2022) and, if injected into adult *D. melanogaster*, can persist for at least a week without causing obvious harm (Duneau *et al*., 2017; Troha *et al*., 2018). Most relevantly, infection by *E. coli* is typically lethal in immunocompromised *D. melanogaster* that lack either a functional peptidoglycan-sensing pattern recognition receptor (Rämet *et al*., 2002) or a functional Imd signaling pathway (Rutschmann *et al*., 2000). While most experiments deliver bacteria directly into the fly by needle, an experiment examining oral exposure found that knockout of the Imd pathway caused *D. melanogaster* to be susceptible to oral infection by ROS-resistant *E. coli* (Ryu *et al*., 2006), demonstrating survival of *E. coli* within the gut, translocation into the hemocoel by unknown mechanisms, and subsequent establishment of infection within the hemocoel with ROS as the main potential barrier. Because ROS production within the gut is elicited as a response to uridine catabolism (E.-K. Kim *et al*., 2020), it is probably not relevant for mirror bacterial infections. Together, these experiments suggest that an immune-evasive generalist like mirror *E. coli* might be able to cause lethal infection in *D. melanogaster* and, potentially, other insect species.

Insects are constantly exposed to environmental bacteria and often ingest them incidentally while consuming other food. Mirror bacteria would be resistant to lytic enzymes, which would facilitate their survival upon ingestion. Insect guts are often colonized by diverse microorganisms (Engel & Moran, 2013; Shao *et al*., 2024). Though the molecular mechanisms regulating the insect gut microbiota are highly varied (Schmidt & Engel, 2021), resistance to lytic enzymes and evasion of innate immune detection would be major advantages that might facilitate mirror bacterial

colonization of the gut in some species. Insect guts often have alkaline regions (Terra & Ferreira, 1994), reaching a pH of 9.5 in *D. melanogaster* (Overend *et al*., 2016) and 12 in some termites (Brune & Kühl, 1996), which could present a substantial barrier to mirrored *E. coli* or other neutrophils. Rapid transit times through the gut may also preclude colonization in some cases (Hammer *et al*., 2017), especially if mirror bacteria prove to be poor adherents (see Section 4.4). Mirror bacterial colonization of the insect gut could disrupt the natural microbiome and cause harm to the insect, as is known to occur during other forms of dysbiosis (Buchon *et al*., 2013; Raymann & Moran, 2018).

An even more concerning possibility is that mirror bacteria might cross the gut epithelium and enter the insect hemolymph. This appears to have occurred, for instance, for *E. coli* in *D. melanogaster* lacking a functional Imd pathway (Ryu *et al*., 2006). It is unclear how frequently natural-chirality commensal bacteria are translocated through the gut epithelium of insects; specialized pathogens such as *S. marcescens* cross the gut epithelium to cause systemic infection (Nehme *et al*., 2007), but likely have specialized adaptations to do so efficiently. Nevertheless, these barriers are likely imperfect and could allow at least low rates of translocation. Translocation may occur even if mirror bacteria do not successfully colonize the gut, though colonization would greatly increase the rate at which it occurs. Mirror bacteria could also enter insects directly through wounds (Subasi *et al*., 2024).

Insect hemolymph is also somewhat different in composition to vertebrate blood. The primary sugar is typically trehalose, rather than glucose, though glucose usually remains present at lower concentrations (Simpson, 2003). Amino acid concentrations are generally high, with both alanine and glycine present at millimolar concentrations (Piyankarage *et al*., 2008; Uchida *et al*., 1990; Zanotto *et al*., 1996). As described in Chapter 1, *E. coli* can use both L- and D-alanine as sole carbon and nitrogen sources, and mutants that can utilize most of the enantiomers of the canonical amino acids have been observed; a strain that can utilize glycine, which is achiral, has also been engineered (Fung *et al*., 2023). It therefore appears plausible that mirrored *E. coli* could replicate within insect hemolymph even without D-glucose catabolism.

As discussed in Box 1.2, D-amino acids can be toxic to natural-chirality bacteria, so L-amino acids could prove similarly toxic to mirror bacteria. Insect hemolymph has higher concentrations of free amino acids than internal compartments in most other animals (e.g., human blood), although these concentrations appear to vary widely both within and between species. However, these concentrations are likely too low to cause growth inhibition of a mirror *E. coli* and other similar mirror bacteria. L-Cysteine may be an exception, as it can inhibit *E. coli* growth at micromolar concentrations in some media (Soutourina *et al*., 2001); it appears plausible, however, that mirror bacteria could quickly adapt to the concentrations of L-cysteine found in most insects (see Box 1.2). Toxicities to other chiral molecules in hemolymph also appear unlikely to prove a significant barrier to infection, given the lack of evidence that enantiomers of common metabolites are toxic to bacteria and the generally low concentrations of any specific metabolite within hemolymph.

Given the above evidence, it is plausible that a mirror *E. coli* or similar mirror generalist bacteria could replicate within many insects, which would likely be unable to mount an effective immune response. Systemic infections by *E. coli* and other typically non-pathogenic bacteria are often lethal

to immunocompromised *D. melanogaster* (Gottar *et al*., 2002; Lemaitre *et al*., 1996; Michel *et al*., 2001; Rämet *et al*., 2002; Rutschmann *et al*., 2000; Schneider *et al*., 2007), and bacterial loads during infection can reach into the hundreds of millions of cells, or roughly 1% of the total fly mass (Duneau *et al*., 2017; Lemaitre *et al*., 1996). Furthermore, bacterial infections in other insects often result in very high pathogen loads (Hu & Webster, 1998; Keshavarz *et al*., 2023; S. Singh *et al*., 2014). Therefore, despite a lack of toxins or deleterious immunological responses that hasten host death during natural infections (Shirasu-Hiza & Schneider, 2007), mirror bacteria may replicate to high titers and prove fatal by depleting critical nutrients, producing metabolic waste products, or creating physical obstructions in the hemolymphatic system.

Insect infection with mirror bacteria could cause ecological damage and increase spread to vertebrates and plants

Insects are among the most abundant terrestrial animals, with a biomass estimated to be 20-fold higher than that of wild vertebrates (Bar-On *et al*., 2018; Rosenberg *et al*., 2023). They play diverse ecological roles, including pollination, seed dispersal, and nutrient recycling (Yang & Gratton, 2014; Noriega *et al*., 2018), and modern declines in insect populations are already an important environmental concern (Wagner, 2020). Even if only a small fraction of insects were susceptible to a mirror bacterial infection, the ensuing ecological damage could be significant (see Chapter 8).

Insects may also spread mirror bacteria to and between other susceptible organisms. Mosquitoes, flies, and fleas, are known to act as vectors for bacterial infection in humans and other vertebrates (Berenger & Parola, 2017; Laroche *et al*., 2018). Mirror bacteria might enter insects that feed on living vertebrates, cadavers, or fecal matter. The insect could then transmit mirror bacteria to other vertebrates through defecation or regurgitation onto food or into wounds and scratches on an animal's exterior, through consumption of the insect by a predator, or through direct injection while feeding off the vertebrate. All but the last mechanism—which requires invasion of the insect salivary glands (Mueller *et al*., 2010)—are plausible even if the mirror bacteria were unable to infect the insect itself, though infection could increase the inoculum that is ultimately delivered to vertebrates. Whether infected by or merely carriers of mirror bacteria, insects could contribute substantially to the environmental dispersal of mirror bacteria, which we discuss further in Section 8.4.

Bacterial plant pathogens can also be spread by insects (Eigenbrode *et al*., 2018; W. Huang *et al*., 2020), especially hemipteran insects which have piercing mouthparts for extracting sap. Again, this requires invasion of the salivary glands, which would likely require the insect itself to be infected by mirror bacteria. We discuss the potential infection of plants by mirror bacteria in Chapter 7.

Mirror bacteria ingested by *C. elegans* could cause fatal infections if able to persist within the worm intestine

Free-living nematode worms are the most abundant animals in soil ecosystems (van den Hoogen *et al*., 2019), where they play an essential ecological role as predators of microbes, including bacteria. Here we focus on the model nematode *C. elegans* because it is the most well-studied nematode.

Bacteria are the primary prey of *C. elegans* (Schulenburg & Félix, 2017), and most pathogenic bacteria infect the worm intestine following ingestion (Ermolaeva & Schumacher, 2014).

The first defense of *C. elegans* against pathogenic bacteria is to simply avoid eating them. *C. elegans* recognizes a large number of chemical cues as attractive or repellent, and these are mostly achiral, volatile molecules (Ferkey *et al*., 2021; Worthy *et al*., 2018). By contrast, most of the water-soluble attractants are chiral, including the amino acids lysine, histidine, cysteine, and methionine (Ward, 1973) as well as biotin (Bargmann & Horvitz, 1991), which could reduce the attractiveness of mirror bacteria as prey. To a nematode, mirror bacteria might "smell" similar to other bacteria, but they may not "taste" the same. Unless they emit a repellent pathogen-associated molecule such as phenazine-1-carboxamide (Meisel *et al*., 2014) or dodecanoic acid (A. Tran *et al*., 2017), mirror bacteria may be recognized through their volatile compounds as potential prey by *C. elegans*.

C. elegans feeds by drawing bacteria suspended in liquid into the pharynx and then trapping them while expelling the liquid (Fang-Yen *et al*., 2009). The nematode has no difficulty ingesting bacterial-sized polystyrene beads, albeit at lower rates than prey (Kiyama *et al*., 2012). This strongly suggests that filtration is primarily dependent on particle size and that nematodes would also ingest mirror bacteria, though plausibly at a reduced rate due to the absence of chiral signals.

Food particles are first processed mechanically by the pharyngeal grinder (Avery & You, 2012), then pass to the acid intestinal lumen where they are enzymatically digested (Dimov & Maduro, 2019). These mechanisms are not perfect, as many natural bacteria are capable of colonizing or infecting *C. elegans* (Garsin *et al*., 2001; Merkx-Jacques *et al*., 2013; F. Zhang *et al*., 2017). This even includes laboratory *E. coli* MG1655, in which restoration of O antigen biosynthesis appears to provide mechanical resistance to pharyngeal grinding (Browning *et al*., 2013). A mirror bacterium would be resistant to antibacterial and digestive enzymes, which would greatly reduce the efficacy of normal digestive mechanisms. The lysozyme ILYS-3, for example, appears to be required in the pharynx for the effective disruption of live bacteria (Gravato-Nobre *et al*., 2016), suggesting that many mirror bacteria would survive the pharyngeal grinder.

While mirror bacterial resistance to digestive enzymes might allow them to survive nematode ingestion, it is not clear that this would suffice to cause infection. Mirror bacteria would likely lack adhesins capable of allowing tight adhesion to the intestinal tract, and, given that passage through the intestines can be very rapid (Ghafouri & McGhee, 2007), mirror bacteria may simply fail to adhere and therefore be expelled by the nematode. If, however, adhesion is not needed for infection, or if non-specific adhesion is sufficient to allow persistence within the gut, then it appears plausible that mirror bacterial infection would result, particularly given that the nematode is so readily colonized and infected by natural bacteria.

Unlike vertebrates or insects, *C. elegans* does not appear to recognize the highly conserved bacterial MAMPs detected by the PRRs of other animals; instead, infections appear to be recognized through a mixture of more specialized pathogen and host damage signals (T. D. Tran & Luallen, 2024). While the signals involved are not well understood, expression patterns for different lysozymes and antimicrobial peptides vary greatly depending on the bacteria that *C. elegans* is exposed to, with much higher expression during exposure to known pathogens as compared to non-pathogens such as

Bacillus megaterium or *E. coli* (Dierking *et al*., 2016), suggesting that the signals are highly specific. Such signals are indeed highly specific in general, and are either molecules associated with specific pathogens—for instance, the toxic metabolite phenazine-1-carboxamide produced by pathogenic strains of *P. aeruginosa* (Peterson *et al*., 2023)—or the effects of toxins that cause specific forms of cellular inhibition (Dunbar *et al*., 2012; Liu *et al*., 2014; McEwan *et al*., 2012; Melo & Ruvkun, 2012). Because mirror bacteria would, in general, produce neither pathogen-specific signals nor toxins, they are unlikely to trigger any of these innate immune responses.

Infection of *C. elegans* by O antigen restored *E. coli* MG1655, which likely lacks specialized means to cause host damage, may provide a reasonable model for a mirror bacterial infection. In this case, bacterial growth causes gut distension, leading to rupture and nematode death (Browning *et al*., 2013). Similar overgrowth of bacteria has been observed in response to infection by *E. faecalis* (Garsin *et al*., 2001), *Salmonella typhimurium* (Aballay *et al*., 2000), and *S. marcescens* (Mallo *et al*., 2002), and appears to be the typical end result of unchecked bacterial replication in the absence of other virulence factors.

Intestinal bloating and associated tissue damage eventually trigger an innate immune response (Radeke & Herman, 2021; J. Singh & Aballay, 2019), though only at a late stage of infection. Notably, the immune response does not appear sufficient to prevent nematode death during infection with O antigen restored *E. coli* MG1655, or in similar bacterial infections. A mirror bacterium would be additionally resistant to many of the downstream antibacterial effectors, including lysozymes (Dierking *et al*., 2016) and C-type lectins (Pees *et al*., 2021). Membrane-disrupting antimicrobial peptides such as caenopores (Roeder *et al*., 2010) may still function—the D-enantiomers of similar peptides in other animals often retain efficacy against bacteria (Wei *et al*., 2009)—but overall, it seems unlikely that *C. elegans* could successfully control a mirror bacterial infection.

C. elegans can learn to avoid both pathogens (J. Singh & Aballay, 2019; Y. Zhang *et al*., 2005) and low-quality food (Shtonda & Avery, 2006). A nematode that encounters mirror bacteria and survives—either because the mirror bacteria cannot successfully colonize the gut or because the immune system successfully cleared the infection—could plausibly learn to avoid consuming mirror bacteria. Avoidance behavior is unlikely to be perfect, but it could greatly reduce ingestion of mirror bacteria.

In summary, *C. elegans* exposed to mirror bacteria could ingest them at similar or somewhat lower rates compared to natural bacteria. Mirror bacteria could be resistant to normal digestive processes and may simply pass through unharmed. If mirror bacteria are able to adhere to or otherwise persist within the intestines, they could probably cause a lethal infection. Avoidance behavior may be somewhat protective against infection if the infectious dose is high and *C. elegans* can learn that mirror bacteria are poor food items before infection occurs.

Substantial uncertainty remains regarding the immune responses of other invertebrates

Beyond insects and nematodes, relatively little is understood about the immune systems of most invertebrates. In many invertebrate species, innate immune receptors have expanded dramatically (Figure 6.2). Unfortunately, nearly all invertebrate innate immune receptors remain uncharacterized

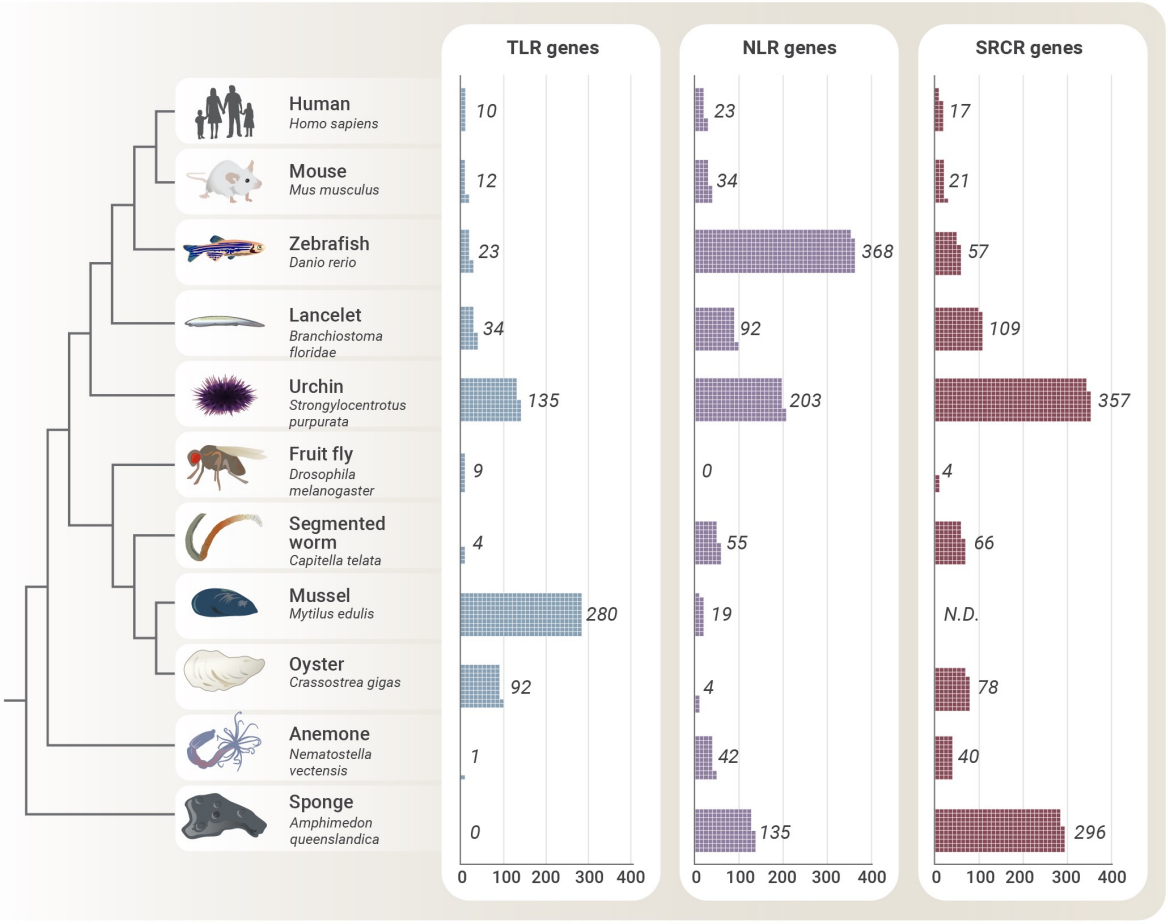

Figure 6.2: The number of PRR genes varies widely across animal species
Waffle plot displaying the number of total known genes for Toll-like receptors (TLRs), nucleotide-binding domain and leucine-rich repeat-containing proteins (NLR), and scavenger receptor cysteine-rich receptors (SRCRs across select animal species. Each square represents one gene. The total number of genes is displayed to the right of each plot. Some PRR gene families have expanded dramatically in certain species. Although nearly all characterized PRRs recognize chiral ligands, most of the proteins encoded by these expanded gene families remain uncharacterized. TLR gene numbers are from (Saco *et al*., 2023). SCSR gene numbers are from (Peng *et al*., 2024). NLR gene numbers are from (Buckley & Rast, 2015), except for *Mus musculus* (Sundaram *et al*., 2024), *Danio rerio* (Howe *et al*., 2016), *Mytilus edulis* (Zhu *et al*., 2022), and *Crassostrea gigas* (L. Zhang *et al*., 2015).

at a molecular level. It is plausible that some invertebrates might have innate immune receptors that can recognize achiral components of pathogens. Moreover, the sheer number of immune receptors in some invertebrate species could increase the likelihood that one or more receptors would cross-react with mirror molecules, thus enabling those invertebrates to recognize mirror bacteria to some degree. Damage detection systems such as those found in *C. elegans* may also offer some protection. However, it seems likely that many invertebrates would have difficulty recognizing and responding to infecting mirror bacteria.

Chapter 7: Plant Infection

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Plants evolved sophisticated immune systems to defend themselves from bacterial pathogens while tolerating low concentrations of bacterial endophytes (Jones & Dangl, 2006; Jones *et al*., 2024). Like animals, plants use a variety of pattern recognition receptors (PRRs) to detect the presence of foreign microbes and release effectors to keep them at low concentrations in the apoplast, the continuous extracellular space through which water and other molecules can move without crossing cell membranes. These receptors are unlikely to recognize mirror biomolecules, and so plants may be susceptible to mirror bacterial infection.

Since plants are poorly studied relative to mammals, and most experiments have focused on the model plant *Arabidopsis thaliana* or a handful of crops, our uncertainty regarding the potential consequences of mirror bacterial infection is considerably higher. Much remains unknown concerning the interactions between plant immunity and bacterial commensals, opportunists, and pathogens. Nevertheless, we have identified two pivotal questions, the answers to which may differ across plant species. First, could mirror bacteria capable of colonizing the apoplast spread beyond their initial site of entry, and second, could infecting mirror bacteria enter the phloem?

Section 7.1 discusses plant immune systems, and shows that a mirror bacterium would likely evade recognition as well as some of the downstream mechanisms used by plants to defend themselves from bacteria.

Section 7.2 discusses localized infections of leaves and roots. In both cases, mirror bacteria could plausibly enter and colonize the apoplast, establishing a local infection. It is unclear if a local infection would be particularly harmful to plants, though it is possible that widespread localized infections could cumulatively cause harm.

Section 7.3 analyzes the potential for infections to spread through vasculature. Mirror bacteria would, by default, lack the extracellular enzymes needed to break down plant physical barriers, which may limit their ability to spread through and colonize xylem. Bacterial entry into the phloem is not common, and primarily occurs via phloem-feeding insects. It is unclear if mirror bacteria could otherwise enter phloem during localized infection. Should it occur, mirror bacterial infection of phloem would likely prove highly damaging or lethal.

Section 7.4 describes countermeasures to protect agriculture. Conventional methods used to control bacterial pathogens would be of limited use against mirror bacteria. Plants could potentially be protected by engineering their existing immune receptors to detect mirror macromolecules, which

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may offer scalable protection for key crop species. Protecting most crop varieties, or even a small fraction of wild plants, is unlikely to be feasible.

This chapter primarily focuses on generalist mirror bacteria such as mirror *Escherichia coli*, which would likely be of greatest scientific interest. It is plausible that deliberate engineering could generate mirror bacteria that were better equipped to colonize plants, or to cause harm to infected plants. Such details are beyond the scope of the chapter.

7.1 Mirror bacteria are likely to evade plant innate immunity

Unlike many animals, plants do not contain circulating professional phagocytes or other immune cells, and they also lack adaptive immunity. Instead, most plant cells rely on innate immune responses that are initiated upon the detection of pathogen molecules by cell surface or intracellular immune receptors. Pattern-triggered immunity (PTI) detects conserved microbial patterns such as bacterial flagellin, and induces a localized immune response featuring release of antimicrobial compounds and proteins, as well as signaling molecules that modify local plant physiology in order to combat and contain the infection (Jones & Dangl, 2006). As with animal immune systems (see Chapter 4 for a discussion on human immunity, and Chapter 6 for other vertebrate and invertebrate animals), cell surface PRRs in plants are expected to poorly recognize enantiomers of their usual targets, precluding direct pathogen targeting. Many downstream defense mechanisms, though not all, would be similarly impaired (Figure 7.1).

Plant immune systems are unlikely to detect mirror bacteria

Plants recognize pathogens through two main pathways: PRRs that detect generic biomolecules found in microbes, and intracellular immune receptors that detect particular pathogen virulence factors (“effectors”) tailored to suppress plant defenses or promote pathogen nutrition (Jones & Dangl, 2006; Katagiri & Tsuda, 2010; Ngou *et al*., 2022). Effectors are protein virulence factors delivered into plant cells by specialized secretions systems, most commonly, the type III secretion system. Mirror bacteria are unlikely to have been created with specialized type III or other secretion systems to deliver intracellular effectors, and so intracellular receptors are unlikely to contribute useful resistance to mirror bacteria (Figure 7.1).

Plant PRRs detect conserved microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) (Chaudhry *et al*., 2021; Dardick & Ronald, 2006). As in animals, these MAMPs are generally chiral (Boutrot & Zipfel, 2017; Schellenberger *et al*., 2019); the only known achiral MAMP, the bacterial lipid cis-11-methyl-2-dodecenoic acid, is a quorum sensing molecule employed by a limited number of bacterial species (Kakkar *et al*., 2015). Although novel receptors continue to be discovered in plants, and some degree of cross-reactivity between a mirror molecule and a normal PRR may be possible, it is likely that most plants would exhibit reduced or even entirely absent initial responses to mirror bacterial infections.

Poor MAMP detection reduces or abolishes downstream antibacterial responses, such as the closure of stomata to prevent pathogen entry (Zipfel *et al*., 2004), the release of defense hormones, and the secretion of antimicrobials and reactive oxygen species (ROS) to kill pathogens. Deleting a single

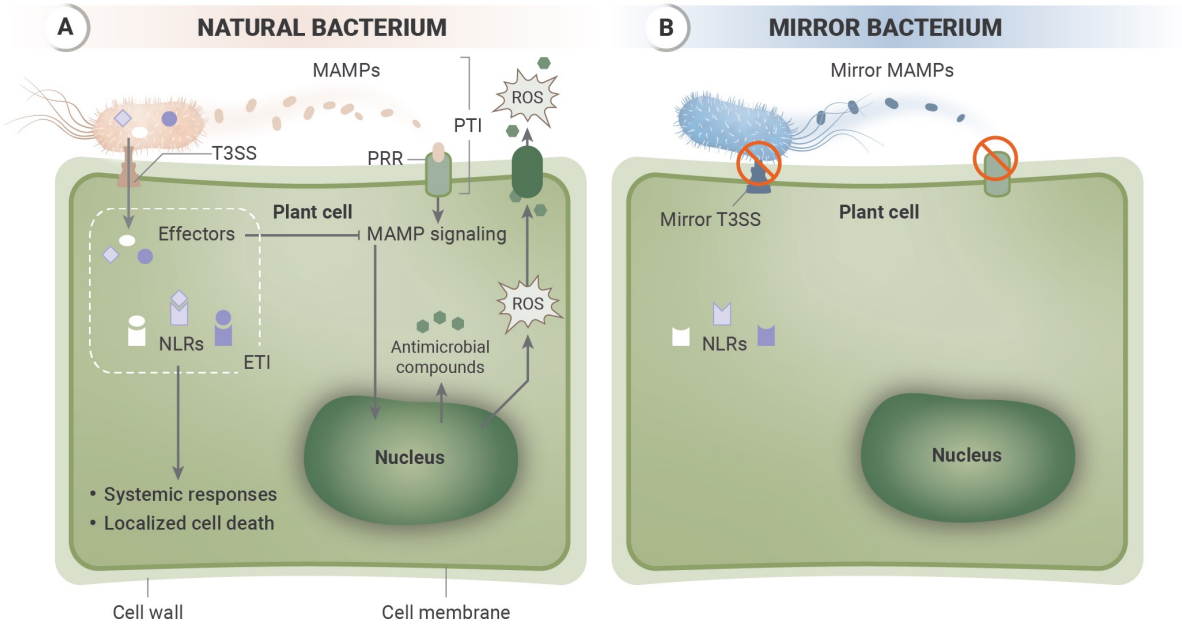

Figure 7.1: Mirror bacteria are likely to evade detection by plant immunity
A. Natural-chirality extracellular[12] bacteria are recognized by two main pathways of plant immunity. Pattern-triggered immunity (PTI) relies on pattern recognition receptors (PRRs) to detect microbial-associated molecular patterns (MAMPs) and induce the release of antimicrobial compounds, reactive oxygen species (ROS), and signaling molecules that modify local plant physiology to combat and contain infection. Effector-triggered immunity (ETI) relies on intracellular immune receptors, such as nucleotide-binding domain and leucine-rich repeat containing receptors (NLRs), to detect bacterial virulence factors ("effectors") that suppress plant immune responses. Effectors are secreted inside of plant cells by specialist Gram-negative plant pathogens via type III secretion systems (T3SS). **B.** Mirror bacteria are unlikely to trigger PTI or ETI. Because nearly all MAMPs are chiral, mirror MAMPs are unlikely to be recognized by plant PRRs. ETI is unlikely to be relevant because mirror bacteria are unlikely to have T3SS or stereospecific effectors capable of interacting with natural-chirality plant proteins.

PRR can result in increased susceptibility to infection by natural phytopathogenic bacteria in experimental models (Ranf *et al*., 2015; Willmann *et al*., 2011), suggesting that impaired recognition by multiple receptors would render plants still more susceptible.

As in animals, it is possible that the later stages of mirror bacterial infection may damage the plant, leading to production of damage-associated molecular patterns (DAMPs). Some DAMPs, such as oligogalacturonides, trigger a significant plant immune response even in the absence of MAMPs (Ferrari *et al*., 2013). However, unlike natural-chirality pathogens, mirror bacteria would not secrete polygalacturonases[13] that generate oligogalacturonides from the plant cell wall, so the pathway is unlikely to be triggered. It is possible that other DAMPs might activate innate immune responses in the absence of MAMPs once sufficient plant colonization has occurred, but how MAMP and DAMP signals are integrated to determine the plant immune response is not fully understood (Saijo & Loo,

[12] Plant immune defenses of the intracellular phloem, discussed in Section 7.3, are poorly understood but may involve some analog of PTI (Jiang *et al*., 2019).
[13] Even if engineered to produce polygalacturonases, the mirror polygalacturonases produced by mirror bacteria are unlikely to target the (chiral) glycosidic bonds within plant cell walls.

2020). Moreover, it is unclear precisely how mirror bacterial infection would damage the plant beyond directly or indirectly depleting it of nutrients, which may not trigger the DAMP response until relatively late in the course of infection, if at all.

Effector-triggered immunity (ETI), typically encoded by disease resistance genes (R genes), recognizes specific pathogen effectors that are delivered into the plant to interfere with MAMP detection (Cui *et al*., 2015; Pfeilmeier *et al*., 2016). ETI powerfully restricts pathogen success: a pathogen that is normally capable of establishing a virulent infection loses that capability if it expresses a single effector recognized by an intracellular immune receptor. Mirror bacteria would not produce such virulence effectors by default and so would go unrecognized by effector-triggered immunity.

Most antimicrobial secondary metabolites and peptides are unlikely to be effective against mirror bacteria

Plants synthesize and accumulate an arsenal of chemical defenses to protect against microbial infection (Tiku, 2020), including small molecules, antimicrobial peptides, and proteases. To be effective against mirror bacteria, these chemicals must be chirality-independent and either reliably released in the presence of the invader or constitutively present in a biologically active form. If immune recognition of mirror bacteria is substantially impaired, the only effective chemical defenses in plants would likely be those that are both active against mirror bacteria and constitutively present.

Such compounds appear to be rare. Antimicrobial proteases (Godson & van der Hoorn, 2021), as chiral enzymes that recognize chiral substrates, would likely be ineffective (see Section 4.2). Most small-molecule toxins generated by plants are phytoalexins, which are synthesized only after pathogens are detected. Phytoanticipins are produced constitutively, but they either exist in an inactive state and are activated by an immune signal or physical damage, or are stored intracellularly in an active form and released upon pathogen detection (Kliebenstein & Kvitko, 2023; Osbourn, 1996). If a mirror bacterial infection becomes sufficiently severe, damage to plant cells may result in the release of these compounds, but they are unlikely to prevent an initial infection.

Most secondary metabolites are also chiral, though there are exceptions, including allicin, catechol, and curcumin (Kocaçalişkan *et al*., 2006; Marchese *et al*., 2016; Zheng *et al*., 2020). However, all of these are phytoalexins, so they would only be helpful if their release was triggered through DAMPs or other means. Cyanogenic glycosides and glucosinolates are activated upon cell damage (Gleadow & Møller, 2014) and the resulting isothiocyanates or hydrogen cyanide would likely act on mirror bacteria, but only if the plant was damaged. Some plants produce achiral metabolites with antibacterial activity that are known to be constitutively present in a biologically active form, including *p*-coumaric acid (González Moreno *et al*., 2022; Lou *et al*., 2012), cinnamic acid (Dorantes *et al*., 2000), and quercetin (Wang *et al*., 2018). Because they are concentrated at plant barriers, they are probably not sufficient to prevent survival of a sufficiently hardy mirror bacterium once it has already entered the plant, but they may help prevent an initial infection.

Plants also produce a diverse range of antimicrobial peptides (Nawrot *et al*., 2014), which often have chiral mechanisms of action (Campos *et al*., 2018) and so would typically not be effective against

mirror bacteria. Thionins, which lyse cellular membranes and exhibit broad antimicrobial activity, provide a plausible exception (Tam *et al*., 2015), although their effectiveness may depend upon potentially chiral interactions with phospholipids (Stec *et al*., 2004). Some antimicrobial peptides are constitutively released by plant cells, but others are either stored intracellularly or synthesized only once a pathogen is recognized (Nawrot *et al*., 2014). Overall, antimicrobial peptides appear analogous to secondary metabolites: most would be ineffective or inactive in the absence of an immune response, but plant immunity is poorly characterized, and there may be exceptions capable of protecting some species (Jones *et al*., 2024).

If triggered, ROS and localized desiccation would retain efficacy against mirror bacteria

While most chemical and enzymatic antibacterials are not expected to be effective against mirror bacteria, other defense mechanisms could still function if triggered. ROS are generally achiral and cause damage through a chirality-independent mechanism that would harm mirror bacteria. However, ROS are also damaging to the plant, so their concentrations within the apoplast are generally tightly controlled (Podgórska *et al*., 2017). Following successful pathogen recognition (Wojtaszek, 1997) or other stressors (Sharma *et al*., 2012), a plant cell may undergo an oxidative burst—a rapid, transient release of large quantities of ROS. Given the anticipated failure of pattern-triggered immunity to recognize mirror MAMPs, this reaction is unlikely to occur. Moreover, many generalist bacteria—like most plant pathogens—express catalase, providing them with some degree of resistance (Fones & Preston, 2012).

Plants also suppress bacterial growth by restricting water availability in the apoplast, much of which is dry and relatively inhospitable to endophytes (Beattie, 2011; Roussin-Léveillée *et al*., 2024). The exact mechanisms are not fully understood, but appear to involve both pattern-triggered and effector-triggered immunity (Z. Liu *et al*., 2022; Wright & Beattie, 2004), and may also be associated with plant cell death (Freeman & Beattie, 2009). Drying of the apoplast could prove effective at halting mirror bacterial growth, particularly since they are unlikely to have water-soaking effectors with which to release water from plant cells. However, local desiccation would only be effective if induced, which appears unlikely.

Finally, plants can reduce concentrations of sugars and other key nutrients in response to bacterial infection (Lemonnier *et al*., 2014; Yamada *et al*., 2016). This could again suppress mirror bacterial growth, but would also likely first require immune activation.

7.2 Mirror bacteria could plausibly establish chronic local infections within leaves and roots

In addition to molecular and chemical immune mechanisms, plants rely heavily on physical barriers to defend their internal spaces from microbial invasion. The external surfaces of plants are not very hospitable to bacteria and are mechanically tough. Only fungal pathogens have evolved mechanisms to traverse these barriers, and bacteria instead usually enter through natural openings, such as lateral root emergence sites, stomata on leaves, hydathodes at leaf margins, or through wounds. Despite these barriers, both specialized plant pathogens and non-specialist commensal bacteria are able to

enter the leaf and root apoplast, and at least occasional mirror bacterial entry into both spaces appears plausible. Given the anticipated failure of plant immunological defenses, it also appears plausible that mirror bacteria could colonize these apoplastic spaces, but the consequences of localized infection are difficult to predict.

This section focuses on whether plant exposure to environmental mirror bacteria could lead to infection. The potential survival and spread of mirror bacteria through the environment, which could result in widespread exposure of plants to mirror bacteria, is addressed more generally in Chapter 8. Different kinds of mirror bacteria could greatly vary in their ability to infect plants. As discussed below, it appears possible that immune evasion could allow a mirror *E. coli* or similar generalist bacteria to colonize many plants, particularly if equipped with the ability to catabolize common sugars. Ecologically invasive mirror bacteria would face strong selective pressures to increase robustness and adaptations could arise quickly (see Section 8.3), increasing both the probability of plant infection and an expansion in host range over time.

Deficits in stomatal closure could allow mirror bacteria to enter leaves

In order to enter a leaf, mirror bacteria first need to attach to and survive on its surface. Leaf surfaces are harsh environments for bacteria, often hydrophobic with fluctuating temperatures and humidity, low nutrients, and exposure to ultraviolet radiation. Despite this, enteric mammalian pathogens such as *Salmonella* and *E. coli* O157:H7 are able to survive on leaf surfaces for long periods of time (Brandl, 2006; O'Brien & Lindow, 1989), causing outbreaks of food-borne illness when contaminating leafy vegetables or other produce (Heaton & Jones, 2008). A mirror bacterium would not necessarily be as hardy, and it would be unlikely to have specialized adhesins with which to attach to leaf surfaces—though this could potentially be (at least partially) remedied by curli or other non-specific adhesins (Jeter & Matthysse, 2005). Nonetheless, prolonged survival of generalist mirror bacteria on leaf surfaces remains plausible for sufficiently hardy strains.

Bacteria present on the surface of a leaf can enter the apoplast through stomata, microscopic pores on the surfaces of leaves that open and close to regulate gas exchange. To keep pathogens from entering, plants rapidly close their stomata when nearby PRRs are activated, a strategy known as "stomatal defense" (Melotto *et al*., 2017). The importance of stomatal defense in preventing disease has been underscored by the vulnerability of model systems with even narrow defects in stomatal defense (Melotto *et al*., 2006; Zeng & He, 2010; Zipfel *et al*., 2004), and by the ubiquity of virulence factors from bacterial pathogens that specifically inhibit the pathways leading to stomatal closure (Kroupitski *et al*., 2009; Melotto *et al*., 2006).

All MAMPs currently known to trigger stomatal closure appear to be chiral (Melotto *et al*., 2017), though many likely remain undiscovered. If most MAMPs that trigger stomatal closure do prove to be chiral, stomata may not reliably close in response to the presence of mirror bacteria, allowing invasion of the leaf interior (Figure 7.2). A chemotactic mirror bacterium may actively approach and enter open stomata due to the relatively greater concentration of nutrients inside the leaf (Matilla &

Krell, 2018); a mirror *E. coli*, for instance, would be attracted by both achiral nutrients and by some natural-chirality hexoses and amino acids (Section 8.2).

Hydathodes, pore-like structures on leaf margins that secrete water and dissolved substances through guttation, provide another potential entry route for bacteria (Cerutti *et al*., 2019). Like stomata, they are defended by PRRs (Paauw *et al*., 2023), which are not expected to recognize mirror bacteria. Finally, mechanical damage—including wounds delivered by herbivores—may provide a third means of entry into the leaf apoplast (Savatin *et al*., 2014).

Generalist mirror bacteria could plausibly grow within the leaf apoplast

Abiotic conditions within the leaf apoplast are generally hospitable to bacterial growth, as reflected by the existence of communities of endophytic commensal bacteria in plant leaves (Chaudhry *et al*., 2021; Wippel, 2023). Temperatures are similar to that of the external environment, and apoplastic fluids are typically mildly acidic (Sharp & Davies, 2009; Tarvainen *et al*., 2023). Osmotic pressure is also unlikely to pose a barrier, given that the water potentials within leaves are typically above

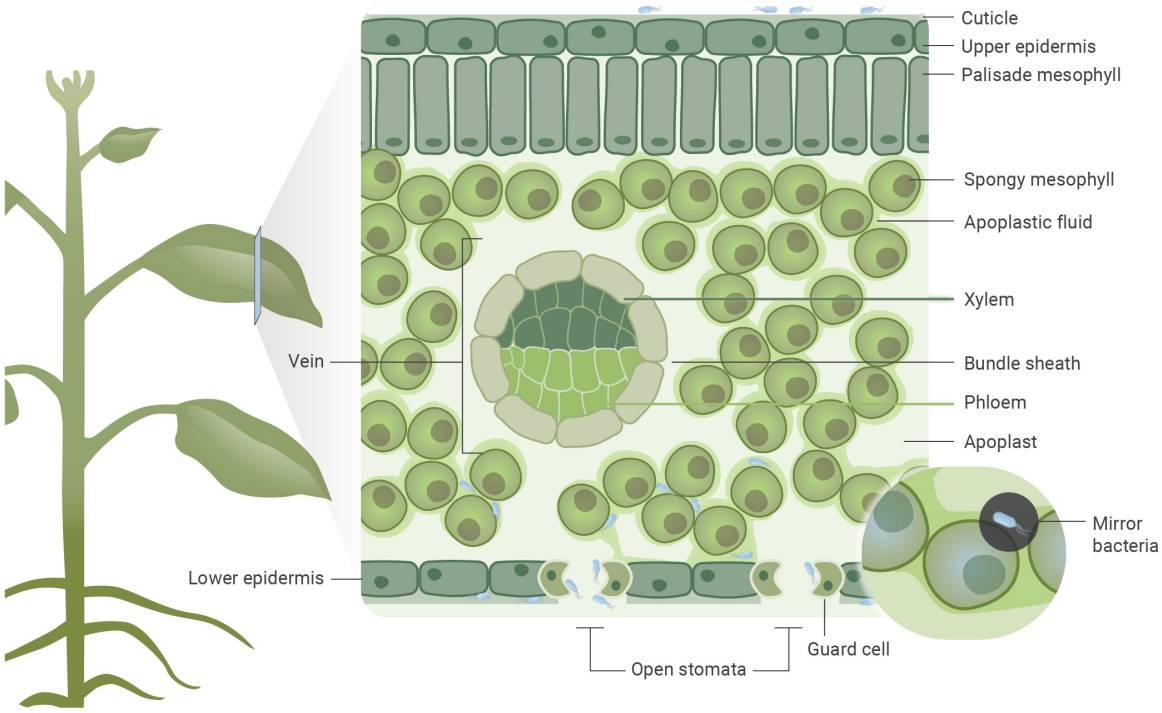

Figure 7.2: Mirror bacteria might enter leaves through open stomata and grow within the leaf apoplast
A single leaf is shown in cross-section. The cuticle and upper epidermis form a physical barrier that would likely prevent mirror bacteria from entering the leaf. The lower epidermis forms a similar barrier, but it is punctuated by microscopic pores called stomata that open and close to regulate gas exchange. Stomata typically close when pathogens are detected through PRRs, but would likely remain open because PRRs will not detect mirror bacteria. As a result, mirror bacteria could move into the apoplast, the continuous extracellular space in the leaf interior. Much of the apoplast is air, but the apoplastic fluid associated with the plant cell walls contains water and some nutrients and might be capable of supporting mirror bacterial growth. Although water and nutrients flow in and out of the leaf via xylem and phloem, it is unclear whether mirror bacteria in the apoplast would be able to pass through the physical barrier formed by bundle sheath cells and enter the vascular system.

−5 MPa (Bartlett *et al*., 2012), equivalent to a water activity of 0.96. Roots and xylem must have even higher water activities in order for water to flow from the roots to the leaves, and activities for fruits and vegetables are also almost always above 0.95 (Schmidt & Fontana, 2008).

While much of the apoplast is filled with air, it contains liquid capable of supporting bacterial growth. Leaf apoplastic fluid contains all of the inorganic nutrients required for bacterial reproduction, as well as amino acids, sugars, and other organic compounds (Gabriel & Kesselmeier, 1999; Lohaus *et al*., 2001; López-Millán *et al*., 2000; O'Leary *et al*., 2016; Rico & Preston, 2008). Concentrations of individual metabolites and minerals within apoplastic fluids are likely highly variable between species. To our knowledge, there has been no systematic attempt to estimate metabolite concentrations across different types of plants. Nevertheless, available measurements suggest that the achiral compounds acetate, γ-aminobutyric acid, citrate, glycine, pyruvate, and succinate are often present at concentrations above 100 µM (Gabriel & Kesselmeier, 1999; O'Leary *et al*., 2016). All of these can be utilized by *E. coli*; acetate, pyruvate, and succinate can serve as sole carbon sources in *E. coli* K12 (see Table 1.1). Concentrations of common L-amino acids may also be around 100 µM (O'Leary *et al*., 2016; Rico & Preston, 2008) and many could provide nutrients for mirror bacteria; L-alanine in particular could be a sole carbon and nitrogen source for a mirror *E. coli* (see Chapter 1). L-malate, which could also be catabolized by a mirror *E. coli*, is often present at around 1 mM concentrations (López-Millán *et al*., 2000; O'Leary *et al*., 2016). In aggregate, it appears plausible that these nutrients could be sufficiently abundant to permit growth of a mirror *E. coli*.

As with other metabolites, apoplastic concentrations of sugars have not been systematically measured, and existing data is highly variable for individual sugars. Sucrose concentrations are typically between 0.1–4 mM (Aked & Hall, 1993; López-Millán *et al*., 2000; Nadwodnik & Lohaus, 2008; O'Leary *et al*., 2016; Oner-Sieben & Lohaus, 2014; Tetlow & Farrar, 1993), while concentrations of sorbitol or mannitol can be in the tens of millimolar (Nadwodnik & Lohaus, 2008). Concentrations of glucose and fructose were measured to be below 50 µM in *Arabidopsis* (O'Leary *et al*., 2016), but 16 mM and 4.5 mM respectively in sugar beet (López-Millán *et al*., 2000). In some circumstances host cell wall invertases could increase hexose concentration in the apoplast, such as in immature leaves (Ruan, 2014) or in response to pathogen infection (Biemelt & Sonnewald, 2006).

These sugars are chiral and so would not be accessible to an exact mirror-image of an *E. coli*. However, as discussed in Chapters 1 and 3, systems capable of L-glucose utilization do exist in a few natural bacteria and if implemented within mirror bacteria would allow D-glucose catabolism. Isomerases interconverting L-fructose and L-glucose are already known (Park *et al*., 2007), as is a dehydrogenase capable of converting L-mannitol to L-fructose (Pail *et al*., 2004), while L-sorbitol could likely be catabolized by incorporating a known pathway from *Stenotrophomonas maltophilia* (Brechtel *et al*., 2002). There are no known enzymes capable of hydrolyzing L-sucrose, although it is possible that a suitable glycoside hydrolase could be discovered, evolved, or designed. Engineering mirror bacteria to consume common chiral sugars—which could be motivated by a number of scientific or practical considerations—might therefore increase the risk of apoplastic colonization.

The consequences of generalist mirror bacterial infection of leaves are unclear

Because mirror bacteria are expected to resist constitutively released plant antibacterials and avoid activating the PTI or ETI responses, their mortality rate within leaves could plausibly be low. Nutrients within apoplastic fluid could support growth, even for mirror bacteria that are unable to catabolize sucrose or other common sugars. Insofar as this growth rate is more rapid than the death rate, which is expected to be low due to impaired immune recognition, the mirror bacterial population would expand.

It is difficult to predict the possible consequences of a localized mirror bacterial infection as there are no obvious natural analogs. Leaves typically contain commensal bacteria, but these endophytes usually remain at low concentrations of 10^3 CFU/g (Hallmann, 2001). Given the availability of nutrients with apoplastic fluid, it appears likely that endophyte populations are directly or indirectly kept at low levels by the host immune response (Hacquard *et al*., 2017). For instance, *Arabidopsis* plants in which multiple immunological pathways have been knocked out can host endophytic populations more than a hundred-fold greater than those in healthy plants (Chen *et al*., 2020), and exhibit a density-dependent immunological response to inoculations with non-pathogenic bacteria (Miebach *et al*., 2024).

By evading these responses, local mirror bacterial concentrations could potentially become multiple orders of magnitude greater than those of typical commensals. Indeed, pathogenic bacteria that release effectors capable of suppressing the immune response can likewise reach high concentrations in leaves (Hirano & Upper, 2000). However, such bacteria generally cause direct harm to the leaf through both intracellular effectors injected into plant cells and/or through extracellular enzymes that damage plant tissue, often in turn providing the bacteria with additional nutrients. They also trigger strong immune reactions from the plant itself, including localized cell death through the hypersensitive response (Balint-Kurti, 2019). Mirror bacteria could not cause harm through any of these mechanisms, and therefore the consequences of infection cannot be predicted on the basis of those caused by natural bacterial pathogens.

Instead, it appears most likely that mirror bacterial populations would expand until growth became limited by nutrients. Given the low concentrations of accessible organic compounds compared to the relatively high concentrations of inorganic nutrients such as iron (López-Millán *et al*., 2000; O'Leary *et al*., 2016) and phosphorus (Gabriel & Kesselmeier, 1999; Lohaus *et al*., 2001; Mimura *et al*., 1992; O'Leary *et al*., 2016), organic carbon would likely prove limiting, causing accessible sugars and organic acids to become depleted in the apoplast. The extent to which this would be harmful to the leaf is unclear. While the leaf apoplast does contain some sugars and other organic nutrients, these comprise only a very small fraction of the total carbon metabolite pools within a leaf, and organic nutrients typically move between cells through symplasmic rather than apoplasmic pathways.

The primary exception is phloem-loading. In many plants sucrose is exported apoplastically within the minor leaf veins, then imported into neighboring phloem sieve elements and companion cells (Braun, 2022). The minor leaf veins are likely inaccessible to mirror bacteria, so even in apoplastic phloem loaders the consumption of photosynthetic production would be limited. There are a few known exceptions: in many deciduous trees, especially sugar maples, but also in plants such as grape

(Walker *et al*., 2021), organic sugars are transported from starch reserves in the roots through xylem to support leaf growth in the spring. As a result, apoplastic sugars can reach concentrations as high as 30–150 mM sucrose (Holgate, 1950; Rapp *et al*., 2019). If metabolically accessible to mirror bacteria, these levels could allow local sugar-limited populations to expand rapidly, potentially consuming enough of either organic or inorganic nutrients to trigger systemic changes in nutrient allocation that may stunt the growth of the plant. However, even this scenario would likely require large fractions of most leaves to be colonized.

Apoplastic sucrose and malate both play important roles in the metabolism and regulation of guard cells (Kang *et al*., 2007; Lawson & Matthews, 2020; Santelia & Lawson, 2016), which unlike other plant cells lack plasmodesmata and so must import organic compounds from the apoplast. Organic compounds in the apoplast also provide nutrients to endophytes, and help regulate apoplastic pH and osmotic pressure. A localized mirror bacterial infection might therefore disrupt leaf homeostasis and reduce productivity, but this is highly speculative, and plants might substantially vary in their susceptibility to harm.

A key overarching question is the extent to which localized mirror bacterial infection could harm the plant as a whole. It is difficult to see how chronic local infections in a limited number of leaves would induce highly deleterious consequences for a plant. Plants routinely lose individual leaves to herbivores or other injuries; a localized mirror bacterial infection might not be very disruptive to an individual leaf, or simply result in the leaf being shed. However, the simultaneous and chronic infection of many leaves could cumulatively stunt plant growth by reducing the flow of sugars to roots and shoots, especially in species with seasonally higher concentrations of organic nutrients in the apoplast. Cumulative local leaf stresses induced by mirror bacterial infection could theoretically cause maladaptive plant behaviors by inducing global stress responses and systemic nutrient redirection, perhaps analogous to the formation of leaf galls that redirect resources by bacterial pathogens such as *Rhodococcus fascians* (Stes *et al*., 2013), but this is highly speculative given that mirror bacteria could not similarly reprogram the plant cells. Further research on whether leaf-specific apoplastic disruptions can trigger systemic responses could help clarify the extent to which localized mirror bacterial infections might be harmful to the plant as a whole. Persistent localized plant infections, even if initially benign, would furthermore provide many opportunities for the evolution of more virulent mirror bacterial strains.

Mirror generalist bacteria within soils might infect plant roots

As discussed in Chapter 8, mirror bacteria would resist most forms of predation and antibacterial weapons, greatly facilitating survival in, and colonization of, soils. The rhizosphere, the region surrounding the roots of a plant, features a distinctive microbiota that is strongly influenced by plant secretions (Baker *et al*., 2024). Root exudates provide bacteria with sugars; predominantly glucose and fructose, organic acids, including citric, succinic, and malic acids (Kamilova et al., 2006); and antibiotic compounds to shape the community composition (Bais *et al*., 2006). Rhizospheric bacteria also produce antibiotic compounds and lytic enzymes to harm competitors and can suppress plant infections by limiting the growth of pathogens (Beneduzi *et al*., 2012; Doornbos *et al*., 2012). Mirror bacterial resistance to most antibiotics and all lytic enzymes, along with resistance to most predatory

organisms (Section 8.1), could aid colonization of the rhizosphere despite competition for nutrients from the natural microbiota.

Plants host diverse endophytic communities that largely derive from the rhizosphere, some of which enter the plant (Figure 7.3). Passive penetration can occur through cracks at lateral root emergence sites, at root tips, or through damage created by other microorganisms (Hardoim *et al*., 2008; Reinhold-Hurek & Hurek, 1998). Emergence sites are defended by root border cells, which produce a mucilage rich in antimicrobial proteins and phytoalexins to prevent bacterial entry (Driouich *et al*., 2013). However, root border cell release and mucilage secretion are both inducible upon pathogen detection, and consequently are likely to prove less effective at blocking mirror bacteria.

Further evidence that bacteria can enter roots comes from studies of *E. coli* and *Salmonella*, which find they can be taken up from soils and hydroponic solutions into plant roots (Alegbeleye *et al*., 2018; Hirneisen *et al*., 2012; Perulli *et al*., 2021). Studies of microplastic beads similarly suggest that, at least within wheat and lettuce, bacteria-sized beads can be internalized through roots (Li, Luo, Li *et al*., 2020; Li, Luo, Peijnenburg *et al*., 2020; Solomon & Matthews, 2005). While these studies generally focus on small, herbaceous plants under conditions that are not always reflective of those in the natural environment or in agricultural settings, they nevertheless suggest that if mirror bacteria were present in soil, they would likely be internalized into the roots of many plants at least occasionally, and perhaps frequently.

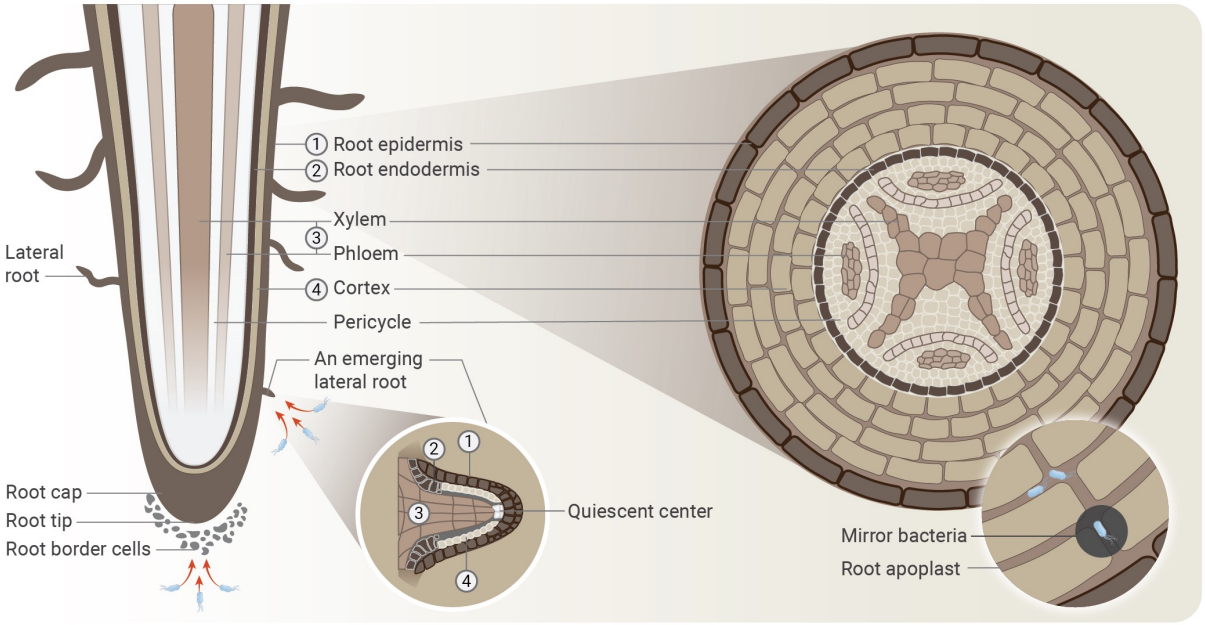

Figure 7.3: The anatomy of a root could facilitate mirror bacterial colonization
An idealized root is shown in longitudinal cross-section (left) and horizontal cross-section (right). The root epidermis covers the outer surface of the root. Cracks in the epidermis at lateral root emergence sites, the root tip, or sites of damage could permit mirror bacteria in the rhizosphere (the microbe-rich soil zone near root systems) to enter the cortex. The cortex is composed of loosely packed cells with large intercellular spaces and apoplastic fluid and could plausibly be colonized by mirror bacteria. In order to reach the xylem and phloem, water and nutrients from the cortex must pass through the interiors of endodermal cells, a barrier that would likely prevent most mirror bacteria in the cortex from reaching the vascular tissue.

Endophytic and pathogenic bacteria are further able to penetrate the root endodermis and pericycle to reach the root xylem, but likely make use of cell wall degrading enzymes to do so (Compant *et al*., 2010). Given that mirror bacteria would lack such enzymes, the extent to which they could further penetrate roots is less clear, although reports of plastic microbead and human pathogen transport again suggests that it may be possible in some plants (see Section 7.3).

Plant roots are typically home to a diverse array of endophytic bacteria (H. Liu *et al*., 2017) and are generally hospitable to bacteria. There have been few studies of root apoplastic fluid (Dragišić Maksimović *et al*., 2014), but like root exudate, xylem sap (discussed in the next section), and leaf apoplastic fluid, it contains variable concentrations of organic acids, simple sugars, and amino acids that could support mirror bacterial growth in many cases. Given that mirror bacteria suffer from reduced mortality from plant immunological responses as well as from antibacterials originating from root border cells and competing bacteria, it is plausible that mirror bacteria could colonize root apoplasts.

As with a localized leaf infection, it is unclear whether colonization of the rhizosphere or localized infections of the root cortex would prove harmful to plants. The consumption of organic nutrients could modify local redox potentials and pH. Metabolites released by plants play an important role in shaping endophytic and rhizospheric microbial communities (Badri & Vivanco, 2009; Shi *et al*., 2011), which in turn can facilitate plant nutrient uptake (Singh *et al*., 2022) and help protect against pathogens (Raaijmakers et al., 2009). Organic acids in particular play important roles in the acquisition of phosphate and micronutrients (Ryan *et al*., 2001) as well as the tolerance of aluminum (Ma *et al*., 2001). Whether the consumption of metabolites by mirror bacteria could disrupt these or other root processes, and if so, the extent to which such disruptions would harm plants, is unclear. Mirror bacteria might disrupt soil microbial communities more generally, causing soil degradation and thereby harming plant growth and productivity in both farmed and natural environments (see Section 8.5).

7.3 The extent to which mirror bacteria could spread through and colonize plant vascular tissue is unclear

The xylem and phloem are the two main components of the vascular system in plants (Figure 7.4). The xylem, which is part of the apoplast, primarily functions to transport water and dissolved minerals from the roots to the rest of the plant via dead, hollow cells, using transpiration and root pressure. The phloem is a bidirectional transport system composed of living cells that moves sugars, amino acids, and other organic compounds produced through photosynthesis from leaves to other parts of the plant, including roots, fruits, and developing tissues. Apoplastic pathogens that enter the xylem or phloem can sometimes spread widely through the plant. A key question is whether localized mirror bacterial infections might do the same, as such spread could have very negative consequences to the plant.

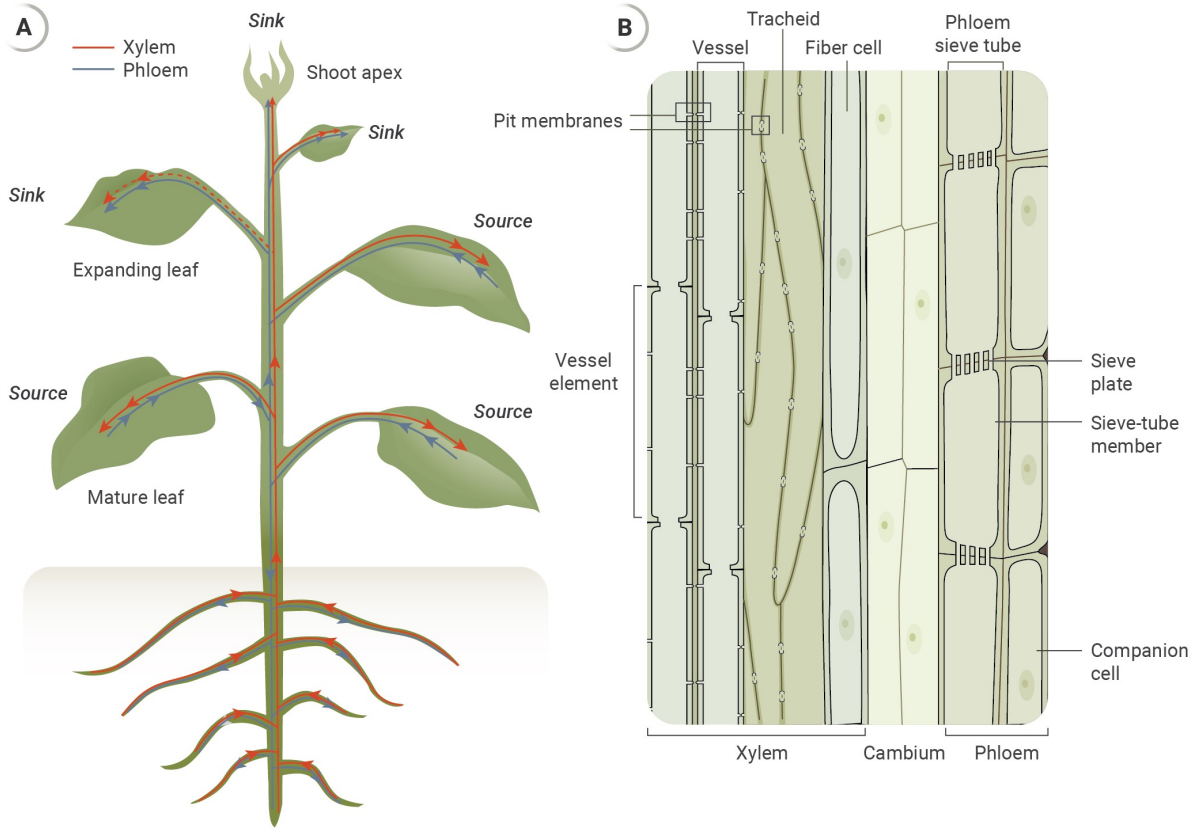

Figure 7.4: Plants transport water and nutrients via xylem and phloem
A. Roots absorb water from soil, which is transported via the xylem through the stem to the leaves and shoot apex. Sources (mature leaves) produce sugars and other photosynthates which are transported via the phloem to sinks, including shoots, young leaves, and roots. Unlike xylem, flow in the phloem can be bidirectional. **B.** Longitudinal cross-section of an idealized vascular bundle from an angiosperm. The xylem is composed of vessel elements and tracheids, which are dead, hollow cells. Vessel elements are stacked end-to-end to form vessels that permit both water conduction and the free movement of bacteria. Vessels are connected to each other and to tracheids by pit membranes that allow water conduction, but the pits are too small to allow free movement of bacteria. The phloem is composed of sieve tube elements and companion cells. Sieve tube elements are elongated, live cells that lack a nucleus and most organelles. They are arranged end-to-end to form sieve tubes and connected by sieve plates that allow fluid and bacteria to freely flow through the tube. Each sieve tube element is connected to a companion cell via plasmodesmata. Xylem and phloem are separated from each other by the cambium, a layer of actively dividing cells.

Mirror bacteria may or may not spread from the roots through xylem

The xylem, a key component of the plant vascular system, is primarily composed of two types of specialized cells: tracheids and vessel elements. While tracheids are found in all vascular plants, vessel elements are unique to angiosperms. Both cell types undergo programmed cell death and lignification as part of their maturation process, resulting in hollow, reinforced structures optimized for water transport. In angiosperms, vessel elements align end-to-end to form vessels, which can range from less than a centimeter to several meters in length (Jacobsen *et al.*, 2012). Lateral connections between vessels, and between vessels and tracheids, are facilitated by pit membranes. These membranes contain pores that are typically less than 100 nm in diameter (Choat *et al.*, 2008),

which permit water movement while theoretically restricting the passage of larger particles, including microorganisms. The primary function of the xylem is to enable the transpiration-driven passive transport of water and dissolved inorganic nutrients from roots to aerial parts of the plant at velocities ranging from approximately 0.3–10 mm/s (Tyree & Zimmermann, 2011; Windt *et al*., 2006).

Pathogenic bacteria can invade and spread through xylem. Being too large to readily pass through the pit membranes, it is generally assumed that xylem-colonizing bacteria utilize cell wall degrading enzymes to travel between these regions (Pérez-Donoso *et al*., 2010; Reinhold-Hurek & Hurek, 2011; Roper *et al*., 2007). The velocity of xylem sap is greater than typically achievable by flagellated bacteria (Milo & Phillips, 2015), so successful colonization requires adhesion to the xylem vessel walls. Movement upstream is thought to occur through twitching motility (De La Fuente *et al*., 2022).

Mirror bacteria would lack cell wall degrading enzymes, which should render them unable to degrade pit membranes. They would also lack specialized adaptations to adhere to, and form biofilms within, xylem, impairing their ability to move against the flow of xylem sap.

Nevertheless, passage through xylem appears to be possible in many plant species (Ryser *et al*., 2009). Studies of microplastic beads indicate that bacterial-sized beads can be internalized through roots and transported to leaves, at least in wheat and lettuce (Li, Luo, Li *et al*., 2020; Li, Luo, Peijnenburg *et al*., 2020; Solomon & Matthews, 2005). Other studies have found that the human enteric pathogens *E. coli* and *Salmonella* can be internalized through roots and transported to leaves in *Arabidopsis* (Cooley *et al*., 2003), barley (Kutter *et al*., 2006), lettuce (Franz *et al*., 2007; Solomon & Matthews, 2005; Solomon *et al*., 2002), parsley (Lapidot & Yaron, 2009), and tomato (Guo *et al*., 2002); at least in *Arabidopsis*, such movement appears to require flagellar motility (Cooley *et al*., 2003). In rice, maize, and other monocots and dicots, rhizobial bacteria enter the roots and are transported to the leaves in large numbers, although they might express cell wall degrading enzymes (Chi *et al*., 2005; Lamb *et al*., 1996). Given that both microplastic beads and the human enteric pathogens definitely lack such enzymes, these findings suggest that a mirror bacteria could potentially travel through xylem in at least some cases.

It is unclear how such transport occurs. One possibility is that bacterial-sized “holes” in pit membranes form often enough to create the occasional unobstructed path from roots to leaves. It is similarly unclear whether such transport can occur in other plants; studies have found successful invasion of crop roots by *E. coli* and *Salmonella* without subsequent translocation to leaves, including in spinach (Hora *et al*., 2005), cabbage (Wachtel *et al*., 2002), and in apple and nectarine trees (Perulli *et al*., 2021). Given the limited evidence available—which is almost entirely restricted to young herbaceous plants—it is difficult to say anything beyond that mirror bacterial passage through xylem could plausibly occur in at least some plants.

In addition to entry through roots, direct entry into the xylem could occur through wounds, particularly those created by insects; entry from leaves might be possible in some cases, particularly if damage is created by other pathogens. Movement of mirror bacteria against the flow of xylem sap, however, appears unlikely. This suggests that they would generally not be able to spread from an infected leaf into other branches or down to the roots absent the evolution of coordinated

xylem-transiting capabilities. However, it must be noted that *E. coli* and *Salmonella* are not thought to colonize xylem. The intrinsic immune evasiveness of mirror bacteria could facilitate xylem colonization. Mirror bacteria that enter roots and become trapped at a pit membrane could potentially reside there for an extended period while increasing in number, affording more opportunities to pass through in the event of a breach.

Generalist mirror bacteria could plausibly colonize accessible regions of xylem

While physical barriers and sap flow may make it difficult for a mirror bacterium to access xylem, any regions that are accessible would plausibly have favorable conditions for growth. Xylem sap is generally considered to be nutrient-poor (Fatima & Senthil-Kumar, 2015), but concentrations of nutrients can be highly variable. Measured glucose concentrations, for instance, vary from 20 μM (Zuluaga *et al*., 2013) to 20 mM (López-Millán *et al*., 2000; Zuluaga *et al*., 2013), while concentrations of organic acids such as malate and citrate can likewise vary from below 5 μM (Anguita-Maeso *et al*., 2021; Fernandez-Garcia *et al*., 2011) to several millimolar (Kasper *et al*., 2022; López-Millán *et al*., 2000; Prima-Putra & Botton, 1998). Amino acid concentrations are also highly variable, with individual amino acids often in the 10–100 μM range but sometimes over 10 mM (Anguita-Maeso *et al*., 2021; Fernandez-Garcia *et al*., 2011; Nakamura *et al*., 2008; Prima-Putra & Botton, 1998; Zuluaga *et al*., 2013). Given that a mirror generalist bacterium could likely catabolize many organic acids, some amino acids, and (with straightforward engineering) glucose and other simple sugars, it seems plausible that mirror bacteria would be broadly capable of replicating within xylem. In the absence of immune recognition and an effective immune response, this could allow colonization of the xylem.

Growing mirror bacterial colonies could plausibly create physical blockages in pit membranes, the likelihood and consequences of which are difficult to assess. A potentially relevant analog is *Xylella fastidiosa*, a xylem-restricted gamma-proteobacterium with a broad host range (European Food Safety Authority, 2020; Rapicavoli *et al*., 2018). While a benign commensal in many plants, *X. fastidiosa* acts as a pathogen in others, causing symptoms that appear to result from vascular occlusions impeding water transport (Chatterjee *et al*., 2008; Sun *et al*., 2013). Similar phenotypes could result from mirror bacterial infections of the xylem. However, unlike *X. fastidiosa*—which can use cell wall degrading enzymes to travel through xylem (Roper *et al*., 2007)—mirror bacteria may be restricted to a small fraction of xylem and consequently limited in their ability to harm the plant. If most of the xylem were accessible to mirror bacteria, whether due to physiological quirks of the plant, slow spread over a long time period, or—like *X. fastidiosa*—by being spread through insect vectors, it appears plausible that a systemic infection could eventually greatly reduce flow of water through xylem, damaging the plant.

Entry into phloem appears difficult

The phloem consists of sieve tubes, elongated sieve element cells positioned end-to-end and connected both vertically and laterally by sieve pores 0.4–15 μm in width (Bussières, 2014; Esau & Cheadle, 1959; Mullendore *et al*., 2010; Thompson & Wolniak, 2008). Phloem sap passively flows from leaves to sink tissues—roots, buds, seeds, and flowers—at around 0.05–0.4 mm/s (Fisher, 1990;

Liesche *et al*., 2013; Windt *et al*., 2006). Unlike xylem sap, the flow of phloem sap can be bidirectional. For instance, phloem sap flows into budding leaves to provide nutrients, but once the leaves mature they become phloem sources rather than sinks (Will *et al*., 2013). A mirror bacterium able to enter and survive within the phloem would therefore likely be freely transported from mature leaves toward sink tissues, including budding shoots and leaves.

Entry into phloem is more challenging than xylem, and appears only likely to occur in rare or exceptional circumstances. Entry requires first breaching the protective bundle sheath cells and then companion cells or sieve elements. While generalized plant pathogens that have broadly overcome plant defenses occasionally enter phloem (Gao *et al*., 2016; Mensi *et al*., 2014; Roos & Hattingh, 1987), all known plant pathogens that primarily inhabit phloem rely on phloem-feeding insect hosts for entry (Jiang *et al*., 2019). Infected insects could potentially deliver mirror bacteria into phloem, though only if the mirror bacteria can reach the insect salivary glands and grow to sufficiently high abundance prior to incapacitating the insect.

Entry into phloem could occur through other types of wounds (Savatin *et al*., 2014). Phloem-localized proteins and callose deposition are believed to occlude sieve plates and thus restrict movement to the rest of the phloem (Bendix & Lewis, 2018), though this remains poorly understood.[14] Nevertheless, there have been reported observations of bacteria entering the phloem while colonizing other plant tissue, including both plant pathogens *Xanthomonas* (Mensi *et al*., 2014; Wang *et al*., 2018) and *Pseudomonas syringae* (Gao *et al*., 2016) as well as human enteric pathogens *Salmonella* (Gu *et al*., 2011) and *E. coli* (Deering *et al*., 2011). If confirmed, especially for bacteria lacking cell wall degrading enzymes, mirror bacteria may be capable of similar entry.

Studies performing metagenomic sequencing of phloem exudate have reported finding nucleic acids corresponding to bacterial endophytes of the leaves and roots in stone fruit and citrus (Eichmeier *et al*., 2019; Y. Wu *et al*., 2020). This could be because living bacteria are occasionally translocated into the phloem before being killed by the immune response or by osmotic effects. Alternatively, these results may instead be explained by transport of nucleic acids from dead apoplastic bacteria into the phloem, or from sample contamination, a common occurrence due to the challenge of obtaining clean samples of phloem (Broussard *et al*., 2023).

If endophytes do occasionally enter into the phloem of some plants without being vectored by insects, mirror bacteria may be capable of doing the same unless cell wall degrading enzymes are required, which is possible. Given how little is known about the presence of natural-chirality bacteria in the phloem outside of the phloem-limited pathogens, more research would be needed to determine whether entry into the phloem occurs commonly, infrequently, or is almost impossible.

Mirror bacteria could plausibly cause a lethal systemic infection if they were to enter the phloem

The immunological defenses of the phloem are poorly understood (Jiang *et al*., 2019; Zhang *et al*., 2020). As a result, it is difficult to assess the extent to which phloem defenses would be evaded by

[14] The role of P-proteins in occluding phloem is particularly controversial (Knoblauch *et al*., 2014; Noll *et al*., 2022).

mirror bacteria. Given the extent to which chiral recognition is required for other plant immune responses, it appears plausible that mirror bacteria could evade phloem defenses. If so, then any mirror bacteria that enter the phloem could trigger unchecked growth in this compartment, with highly deleterious consequences for the plant.

Phloem sap is very nutrient-rich. Sucrose is the primary sugar in the phloem sap of most plants, with typical concentrations over several hundred millimolar (Broussard *et al*., 2023; Dinant *et al*., 2010). Reducing sugars such as glucose and sucrose are typically rare (D. D. Liu *et al*., 2012), while polyols such as mannitol and sorbitol are the primary carbohydrates in some species (Nadwodnik & Lohaus, 2008). Amino acids are the primary source of nitrogen, with total concentrations over 100 mM, including 1–10 mM of alanine (Dinant *et al*., 2010; Hijaz & Killiny, 2014). Organic acids, such as malate, succinate, and citrate, may also be present at millimolar concentrations (Broussard *et al*., 2023; Hayashi & Chino, 1985; Lohaus *et al*., 2000). Phloem thus contains plentiful nutrients for a mirror *E. coli* or a similarly robust mirror bacterium, though accessing the major sugars would require catabolic pathways for sucrose, mannitol, and sorbitol.

Even though the concentrations of organic compounds within phloem sap are high, the overall water potential of around −1 to −2 MPa (Thompson & Holbrook, 2003) should support the growth of most bacteria. *E. coli*, for instance, can grow at sucrose concentrations above 1 M, though rapid changes in concentration can cause most cells to die through plasmolysis (Kawarai *et al*., 2009; Scheie & Rehberg, 1972). Phloem sap is also slightly alkaline (Dinant *et al*., 2010), and temperatures are close to those of the external environment.

As discussed in Box 1.2 of Chapter 1, millimolar concentrations of individual D-amino acids can inhibit bacterial growth. Given the very high amino acid concentrations in phloem sap, it is plausible that amino acid toxicity could reduce or entirely preclude the growth of some kinds of mirror bacteria. However, resistance to these toxicities may be relatively accessible to evolution, such as through inactivation of the permease importing the toxic amino acid into the cytosol or upregulation of an existing detoxifying enzyme.

Given the high nutrient concentrations and likely failure of immunological defenses in phloem, it is plausible that a mirror bacterium able to enter and survive in phloem could replicate unchecked and reach very high concentrations. Mirror bacteria could accumulate in the phloem of roots, shoots, and other sinks, which could cause severe disruption to neighboring tissues by consuming nutrients, releasing metabolic waste products, or simply creating physical blockages.

In addition to the potential for deleterious effects from organic nutrient depletion, the introduction of an aerobic bacterial pathogen into the phloem could be quite harmful to the plant. Unlike animals, plants lack an active circulatory system specialized for distributing oxygen to tissue, and hypoxia within phloem tissue occurs within healthy plants (van Dongen *et al*., 2003). Even if restricted to achiral nutrients, aerobic respiration by mirror bacteria would likely be limited by oxygen, not organic carbon, such that regions of phloem colonized by mirror bacteria could readily become anoxic. Anoxic plant cells must produce ATP through fermentation, which yields much less energy than aerobic respiration per mole of carbon and produces toxic byproducts such as ethanol and lactate (Geigenberger, 2003). Plants vary in their ability to tolerate temporarily anoxic conditions

(Bailey-Serres & Voesenek, 2008), but long-term anoxia would almost certainly disrupt plant cell functionality and lead to cell death (Loreti *et al*., 2016).

Other disruptions are possible. The phloem is continuous with the cytoplasm of adjacent plant cells, so any changes to phloem acidity, redox potential, or composition could disrupt functionality of neighboring cells. Even mirror proteins and other mirror macromolecules from lysed mirror bacteria could be transported through plasmodesmata into neighboring cells (Stadler *et al*., 2005), where they would remain undigested. Large mirror bacterial populations could themselves create physical blockages that prevent flow of phloem into roots, shoots, and other sink tissues.

Disruptions to phloem unloading caused by localized cell death or bacterial overgrowth would stunt or kill roots, shoots, fruits, and other sink tissues. Root death would, in turn, prevent the flow of water and inorganic nutrients back to the leaves, leading to wilting and plant death. Systemic stunting and necrosis are common symptoms of phloem-limited pathogens (Bendix & Lewis, 2018), which provide possible analogs to a mirror bacterial infection. Interestingly, phloem-limited pathogens are typically incapable of aerobic respiration (Christensen *et al*., 2005; Duan *et al*., 2009; Marcone, 2014; Oshima *et al*., 2004) and may be poor analogies for mirror bacteria in other important ways.

Phloem immunological defenses are poorly understood and likely variable between plant species; any attempt to predict the consequences of a putative mirror bacterial phloem infection is therefore necessarily speculative. Nevertheless, if mirror bacteria are able to enter the phloem and establish an infection—which remains unclear—the consequences are likely to be highly deleterious for most plants.

7.4 Countermeasures for agricultural plants

The vast majority of human calories are produced through agriculture, with more than half coming from just four crops: wheat, rice, maize, and soybeans (D'Odorico *et al*., 2014). Crop pests and pathogens, especially fungi, are responsible for yield losses of approximately 25% (Savary *et al*., 2019). Given that fairly mild biotic and abiotic stressors can cause large productivity losses (Boyer, 1982), even relatively mild mirror bacterial infections could plausibly cause significant reductions in yield. Worse outcomes might be possible. Wheat reportedly transports bacteria-sized microplastic beads from its roots to the leaves (Li, Luo, Li *et al*., 2020; Li, Luo, Peijnenburg *et al*., 2020), while rice and maize transport rhizobial microbes in a similar manner (Chi *et al*., 2005; Lamb *et al*., 1996). If these organisms are susceptible to systemic infection, then invasive mirror bacteria could cause substantial losses in the major crops.

Conventional methods of controlling bacterial pathogens would be of limited use against mirror bacteria

As discussed in Chapter 8, a sufficiently robust mirror bacterium could plausibly survive or even colonize many soils and spread widely through animal hosts and human activities. If such a strain were also broadly capable of infecting plants, it could cause unprecedented and widespread agricultural losses. Preventing crop exposure to invasive mirror bacteria through cultural methods

such as quarantines, crop rotations, elimination of alternative hosts, and removal of infected plants is unlikely to be effective. Soil treatments such as solarization, steam heating, fire, or chemical disinfestation could presumably reduce mirror bacteria concentration within soils, but are not feasible at scale, would severely disrupt natural microbiota, and/or may not be able to completely eliminate the bacteria. Even if mirror bacteria were to be eliminated in a field, they could be reintroduced by animals or from windborne dust and debris.

Antibiotics have seen limited use in agricultural contexts as their use is not economical (McManus *et al*., 2002); they are generally only effective at preventing infection and remain active only for a few days (Stockwell & Duffy, 2012). Fungicides, which are far more common in agriculture, may be a better comparison. Many systemic fungicides, which are internalized into the plant and typically transported through the apoplast—phloem mobility is rare—are able to cure and eradicate, rather than merely prevent, fungal infection (Oliver & Hewitt, 2014). However, even if systemic antimirror compounds were identified, they would need to be constantly reapplied to crops to prevent reinfection from environmental reservoirs such as wild plants and untreated soils. The scale of production required to protect crops at scale may therefore prove infeasible.

Copper-based antimicrobial compounds are achiral and comparatively cheap. Due to their phytotoxicity, however, their application is limited to the preventative treatment of leaves: they have no curative or systemic activity (Lamichhane *et al*., 2018).

Biological methods use natural enemies such as predators or microbial antagonists to control pathogens (van Lenteren *et al*., 2018). As discussed in Section 8.1, mirror bacteria could be resistant to most forms of predation and many forms of microbial antagonism, and it is unlikely that existing biological controls would be very effective. More speculative biological controls, such as mirror phages or genetically engineered microbes, are described in Section 8.6. Using such methods to eliminate mirror bacteria from a local environment such as a field appears to be very difficult; containing and eliminating invasive mirror bacteria from the environment more broadly does not appear feasible by any method.

Plants might be engineered to resist infection

While highly speculative, engineering plants to be resistant to mirror bacteria could be a feasible strategy to enable agricultural production at scale despite the presence of an invasive mirror bacterium. One promising strategy would involve engineering immune recognition of certain mirror MAMPs, thus allowing innate immune defenses to trigger in response to mirror bacteria.

Plant PRRs, which are responsible for monitoring the apoplast, are conserved and remain functional when expressed transgenically in a wide range of plants (Lacombe *et al*., 2010). For example, the EF-Tu receptor from *Arabidopsis* protects against bacterial diseases in tomato (Kunwar *et al*., 2018), potato (Boschi *et al*., 2017), and citrus (Mitre *et al*., 2021). If any naturally occurring PRRs have some cross-affinity to mirror bacterial cell wall components—which is plausible, given the diversity of PRRs found in nature (Boutrot & Zipfel, 2017)—then these could be enhanced and transferred to major crops.

Engineering the binding ectodomain of a PRR to recognize unique signatures of mirror bacteria would allow targeting of patterns such as D-peptide multimers that are absent from nature. Although there is not yet any equivalent to engineered mammalian SNIPR receptors in plants (Zhu *et al*., 2022), the anti-flagellin ectodomain of the FLS2 has been successfully evolved to recognize a different peptide (Helft *et al*., 2016), and similar leucine-rich repeat domains have been retargeted by phage and yeast display (Velásquez *et al*., 2017; Wezner-Ptasinska & Otlewski, 2015). Once a suitable ectodomain has been engineered, it could be used to create chimeric PRRs with native intracellular kinase domains. Chimeric PRRs utilizing natural ectodomains have already been engineered and found to work *in planta* (Albert & Felix, 2010; Schwessinger *et al*., 2015; J. Wu *et al*., 2019). Directed evolution of the ectodomain or design of a novel binder specific to mirror peptide multimers followed by engraftment onto an existing PTI receptor could potentially confer mirror-specific recognition and induction of the plant immune response. The XA21 receptor from rice, which provides ETI-level resistance despite being extracellular ETI (Ercoli *et al*., 2022), is a particularly promising candidate for engineered D-peptide multimer recognition. For example, promiscuous D-peptide minibinders that do not bind biomolecules found in plant tissues might be incorporated into the N-terminal half of the native leucine-rich repeat ectodomain scaffold (Yeh *et al*., 2023), likely preserving the signal-transducing interface between binding and transmembrane domains while reprogramming receptor specificity. Encoding several such engineered receptors recognizing a variety of D-peptide multimers could robustly confer immunity to mirror bacteria.

Another possible route might leverage the nucleotide-binding, leucine-rich repeat (NLR) proteins that recognize intracellular bacterial effectors (Kourelis & van der Hoorn, 2018), which have been subject to more extensive engineering than PRRs (Bentham *et al*., 2023; Cesari *et al*., 2022; De la Concepción *et al.*, 2019; Y. Liu *et al*., 2021; Maidment *et al*., 2023; Zdrzałek *et al*., 2024). Most relevantly, nanobody-NLR fusions have been demonstrated to trigger ETI upon recognition by the nanobody, suggesting that this approach can be generalized to recognize any molecular trigger (Kourelis *et al*., 2023). However, invasive mirror bacteria are not expected to enter plant cells, so traditional NLRs would not be useful: the relevant signaling domains would need to be fused to the transmembrane domain of an extracellular receptor, as with XA21.

To compensate for the reduced efficacy of many plant antimicrobials against mirror bacteria, new antimirror molecules might be engineered into plants, much as the transgenic expression of antimicrobial peptides can increase plant tolerance to existing bacterial pathogens (Balaji & Smart, 2012; Hao *et al*., 2016; Zou *et al.*, 2017). For example, the constitutive overexpression of chirality-independent antimicrobial peptides could provide always-on resistance, while inducible effectors triggered by synthetic mirror-specific receptors could provide targeted immunity. Metabolic pathways for anti-mirror small molecules might also be introduced or (for achiral antimicrobials) upregulated. Modifications to limit the availability of key nutrients could provide an additional method to increase plant resistance to infection.

While many of the strategies for engineering heritable resistance to mirror bacteria may appear promising, it is important to note that all of these putative methods of preventing infection are necessarily speculative. They may have unintended and undesired consequences, such as reducing yields, and may not prove entirely effective at preventing mirror bacterial contamination of food.

Developing microbial methods of food production or cultivating simple plants such as duckweeds that are likely to resist mirror bacterial infection might provide a more robust and generalizable defense against threats to traditional agriculture, but these alternatives are almost wholly undeveloped. If engineering heritable mirror resistance in crops is pursued, it would need to be performed separately in each crop species or cultivar. Protecting wild plants and their associated ecosystems would not be feasible.

Chapter 8: Environmental Survival and Spread

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Populations of organisms expand when their reproductive rate exceeds their death rate. The availability of resources and efficiency of conversion into progeny determine rates of reproduction. Resource availability decreases in the presence of competing species. Death rates are governed by natural enemies such as predators and pathogens, as well as by environmental stresses such as desiccation, extreme temperatures, and starvation. When a species' reproductive rate exceeds its death rate, its abundance will increase until resources are exhausted, natural enemies proliferate, or the environment changes. For most species there is an approximate balance between births and deaths, such that populations remain approximately constant or exhibit a seasonal cycle influenced by nutrient availability and predators.

Occasionally, the population of a species introduced into a new environment with fewer natural enemies, including predators, can increase dramatically, sometimes causing great damage to natural and agricultural ecosystems. Even if the introduced species is somewhat less adapted to its abiotic conditions or local resources than native species, it can often expand and cause harm—becoming invasive—due to a low death rate resulting from the absence of natural enemies.

A major concern in this chapter is that mirror bacteria could, by virtue of their altered chirality, escape from many natural enemies and so have a greatly reduced death rate due to predation and parasitism. In addition, as discussed in Chapters 4, 6, and 7, mirror bacteria could plausibly infect and survive in a wide range of multicellular organisms. Nevertheless, mirror bacterial growth rates may be substantially lower than those of natural bacteria, as mirror bacteria would have a reduced capacity to obtain nutrients and would lack other specialized adaptations for most environments encountered. Should the advantages from reduced predation and any benefits from infecting multicellular organisms outweigh the disadvantages in a given environment, mirror bacteria could then colonize that ecosystem.

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Imagine, for example, an environment in which the natural-chirality bacteria, on average, divide once per day and die once per day, thereby maintaining roughly steady numbers. Now imagine a mirror bacterium is introduced that escapes predation and experiences only a quarter of that mortality. Even a two-thirds reduction in the mirror bacterium's growth rate would still exceed its death rate, allowing the mirror bacterium to invade and increase in numbers until the available nutrients are sufficiently depleted. Without effective predation and an accompanying increase in the number of predators, mirror bacterial populations could potentially reach very high densities, with extraordinarily damaging consequences for the environment, agriculture, and human wellbeing.

Section 8.1 discusses the effects of biotic processes on survival. Phage lysis, protist predation, and microbial warfare rely primarily on chiral mechanisms. Mirror bacteria would likely experience reduced rates of predation and would be completely resistant to natural phages, which may allow long-term survival outside of hosts and facilitate transmission between infected multicellular organisms.

Section 8.2 discusses the possibility that a mirror bacterial generalist capable of evading most forms of biological control could invade environments outside of multicellular hosts. Mirror heterotrophic bacteria could be capable of utilizing a number of common nutrients, and though their growth would be impaired compared to that of natural bacteria, escape from natural predators could still enable the colonization of some environments. Such invasive bacteria could plausibly grow until most nutrients are depleted, as most top-down biological controls would be absent.

Section 8.3 describes the possible spread of an invasive mirror bacterium through the environment by hitchhiking on human transportation and animal hosts, or by wind or water, allowing rapid dispersal.

Section 8.4 examines the possible evolution of invasive mirror bacteria. Absent human intervention, there would be little genetic diversity, and mirror bacteria would have no ability to obtain genetic material from natural chirality bacteria, leaving *de novo* mutation as the only source of variation and new capabilities. Nonetheless, evolutionary adaptation under conditions of population expansion into new environments can be rapid. By contrast, we expect there would be little potential for predators to evolve to consume mirror bacteria within any human-relevant timescale.

Section 8.5 describes potential ecological harms that could result from the spread of invasive mirror bacteria, including the extinction of susceptible animals and plants as well as changes to habitats and biogeochemical cycling.

In Section 8.6 we close with a brief discussion of possible countermeasures. With sufficient coordination and resources, certain countermeasures could mitigate some harmful effects, but they appear unlikely to prevent widespread and irreversible environmental harm.

8.1 Mirror bacteria would be inherently resistant to many biological controls

In natural environments, individual bacterial cells often perish from predation by protists and bacteriophages (viruses that infect bacteria, often referred to as “phages”) or are killed by the microbial weapons of competitors (Kirchman, 2018; Pernthaler, 2005; Thingstad, 2000). Mirror

bacteria would likely be largely resistant to protist grazing, entirely resistant to natural phages, and resistant to most weapons of microbial competitors. This inherent resistance could substantially enhance the survival of mirror bacteria in most ecosystems.

Mirror bacteria would be resistant to natural phages

Phages are responsible for a large fraction of bacterial deaths in many environments. There are 10 to 20 phages for every bacterial cell in both soils and natural waters (Cobián Güemes *et al*., 2016), leading to frequent encounters (A. G. Murray & Jackson, 1992). However, not every cell is susceptible to every phage, and natural bacteria have evolved defenses against some of the phages that infected their ancestors. Despite these defenses, bacterial death rates from phages in the best-studied marine environments range from ~0.01 day^{-1} to over 20 day^{-1} (Carlson *et al*., 2022; Mann, 2003; Weinbauer, 2004). Many bacterial populations grow rapidly while they are resistant or otherwise phage-free, then encounter a phage to which they are susceptible and are dramatically reduced in number. For example, cyanobacterial "blooms" created by nutrient influxes in aquatic environments are often ended by phages (Grasso *et al*., 2022; T. D. Harris *et al*., 2024; Mann, 2003). Mirror bacteria would be inherently and completely resistant to every natural phage, likely providing a major fitness advantage.

All viruses parasitize their host's cellular translation system and nucleoside triphosphate pool to produce proteins and new viral genomes. Mirror ribosomes could not produce natural-chirality proteins, nor could mirror nucleotide triphosphates be used to replicate natural-chirality viral genomes. Thus, no natural phage could successfully reproduce within a mirror bacterium. Even infection—the entry of the phage's genetic material into a mirror bacterium—is mediated by chiral phage proteins binding to chiral cellular receptors, and therefore is highly unlikely to occur (Labrie *et al*., 2010).

In summary, natural phages would not be able to infect mirror bacteria, completely removing a major source of bacterial mortality. Only synthetic mirror phages, which do not currently exist, could kill and replicate within mirror bacteria; they are discussed as a potential medical countermeasure in Section 5.3 and as a potential environmental countermeasure in Section 8.6.

Mirror bacteria would probably be resistant to other predators

In addition to phages, bacteria are preyed upon by phagocytic amoebae and other protists, other bacteria, and multicellular eukaryotes including nematodes, arthropods, and rotifers (T. D. Harris *et al*., 2024; Morgan *et al*., 2010; Potapov *et al*., 2022). Mirror bacteria could be harder for predators to track because they would leave trails of mirror-image biomolecules that would not properly bind to the predator's receptors; they could be resistant to being killed even when consumed; and they would provide few nutrients of use to their predators. Animals that ingest mirror bacteria could instead become infected by them (Chapter 6).

We first discuss protists. In marine ecosystems, protists are often the leading cause of bacterial death and the primary control on bacterial abundance (Pernthaler, 2005). Grazing rates by microzooplankton on phytoplankton of ~40% per day are common, with approximately two-thirds of

primary production consumed by this protist-dominated group (Calbet & Landry, 2004); however, which species are primarily responsible for this consumption is much less certain (Frias-Lopez *et al*., 2009; Wilken *et al*., 2023). Although less well-studied than the marine variety (Geisen *et al*., 2017), terrestrial protists are also major consumers of bacteria in soil (Geisen *et al*., 2018; Trap *et al*., 2016).

Bacterial consumption by protists usually occurs through phagocytosis. This is the same process used by the professional phagocytes of the human immune system (Desjardins *et al*., 2005), and so protist phagocytosis would therefore likely suffer from analogous defects to those described in Section 4.2. Protists are highly diverse, and the molecular mechanisms involved in the tracking, recognition, engulfment, and killing of prey are not well-characterized in any organism. This makes it difficult to determine how a given protist would respond to mirror bacteria, particularly given that recognition and killing could still occur to some extent through non-specific or achiral mechanisms.

Box 8.1 discusses phagocytosis of mirror bacteria in the model amoeba *Dictyostelium discoideum*, which is perhaps the most well-studied of all protists. The evidence here suggests that mirror bacteria could be ingested at greatly reduced rates due to the absence of specific prey signals such as folate and peptidoglycan (Pan *et al*., 2018). Whether ingested mirror bacteria would survive phagocytosis is unclear, but it is likely that the undigested mirror bacterium—whether living or dead—would be egested after several hours.

Outside of *D. discoideum*, the molecular mechanisms involved in phagocytosis are less characterized. Prey-seeking based on chiral mechanisms occurs in many protists, which can sense and actively swim toward bacterial exudates[15] such as sugars and amino acids (Spero, 1985; Martel, 2006; Rashidi & Ostrowski, 2019; Roberts *et al*., 2011) and discriminate between prey through the recognition of specific surface compounds (Jürgens & Matz, 2002; Montagnes *et al*., 2008; Shi *et al*., 2021) such as mannose (Wootton *et al*., 2007). Since most recognized surface molecules are thought to be chiral, it is likely that most protists would ingest mirror cells at substantially lower rates. However, protists ingest latex beads and microplastics (Rillig & Bonkowski, 2018) and some protists consume them at rates similar to their consumption of natural bacteria (Pace & Bailiff, 1987), so some phagocytosis of mirror bacteria would likely occur even if chemotaxis were to completely fail.

Once engulfed, bacteria are subjected to acidic conditions and lytic antimicrobial enzymes (Guerrier *et al*., 2017; Shi *et al*., 2021) and, at least in *D. discoideum*, ROS and transition metal poisons as well (Dunn *et al*., 2017). Lytic enzymes are not expected to function, so efficient intracellular killing of mirror bacteria would be likely only if achiral mechanisms were adequate and triggered absent prey signals. It is not clear that this is the case for *D. discoideum* (Box 8.1). Regardless of whether it would be killed or not, the mirror bacterium would not be digested. Most likely either the mirror bacterium or its remains would be egested, but other outcomes are possible if egestion fails. In some cases it may even be possible for living mirror cells to persist within protist food vacuoles like certain

[15] While most solute attractants are likely chiral, there are exceptions. The marine dinoflagellate *Oxyrrhis marina*, for example, is attracted to achiral dimethylsulphoniopropionate (Breckels *et al*., 2011) as well as to chiral amino acids and sugars (Martel, 2006). Volatile compounds are small and often achiral, and so it is plausible that mirror bacteria might "smell" more normal to soil protists than they "taste". The extent to which volatile compounds produced by bacteria influence predatory protists is largely unknown, but one study of three protists and six bacterial species found effects that were strongly dependent on the specific pairing of predator and prey (Schulz-Bohm *et al*., 2017).

Box 8.1: *Dictyostelium discoideum* may not effectively phagocytose and kill mirror bacteria

Dictyostelium discoideum is a soil-dwelling amoeba (or "slime mold") used as a model organism to study phagocytosis, and has often served as a model for human phagocytic cells.

The mechanisms by which *D. discoideum* recognizes prey are not well understood (Dunn *et al*., 2017), but different bacterial species are known to elicit specific transcriptional responses (Lamrabet *et al*., 2020; Nasser *et al*., 2013). The G-protein-coupled receptor fAR1 detects chiral folate and lipopolysaccharide produced by bacteria (Pan *et al*., 2018); one fAR1 knockout strain showed a significant reduction in uptake of the prey bacterium *Klebsiella aerogenes* (Pan *et al*., 2016). Other, as yet uncharacterized, pathways appear to be involved in uptake of *Bacillus subtilis* (Lima *et al*., 2014). There is also evidence for the use of (as yet unidentified) lectin-like receptors in phagocytosis (Vogel *et al*., 1980). It is unclear if any would detect achiral molecules. *D. discoideum* can also phagocytose latex beads, although mutants that can phagocytose beads but not bacteria are known (Vogel *et al*., 1980), suggesting that latex bead uptake is driven by hydrophobic interactions that are absent for many natural bacteria and therefore may not be that relevant for their mirror counterparts. The extent to which *D. discoideum* would engulf mirror bacteria thus remains unclear, but it appears likely that engulfment would occur at a substantially lower frequency compared to natural bacteria.

Once the outer membrane engulfs a food particle, it becomes enclosed within a compartment called a phagosome. During phagosomal maturation the pH decreases to 3.5–4.5 (Gopaldass *et al*., 2012; Marchetti *et al*., 2009), and lytic enzymes, oxidases, and metal transporters are delivered, contributing to a bactericidal environment (Dunn *et al*., 2017). During late maturation nutrients are absorbed and enzymes retrieved. Undigested material is then egested from the cell; the entire process occurs over a few hours. Phagosomal trafficking, acidification, the delivery of lysosomal hydrolases, and the final expulsion of undigested material all proceed normally during the phagocytosis of latex beads (Gotthardt *et al*., 2002; Padh *et al*., 1993) and so would likely also occur in response to the ingestion of a mirror bacterium.

D. discoideum deploys both chiral mechanisms, such as lytic enzymes; and achiral mechanisms, such as reactive oxygen species (ROS) and transition metal poisons (Barisch *et al*., 2018; Hao *et al*., 2016), to kill bacteria. Knockout experiments suggest that reducing the efficacy of lysosomal enzymes greatly impairs intracellular killing of *Escherichia coli* and other bacteria, while the effect of removing ROS generation or increasing phagosomal pH appears comparatively modest (Crespo-Yanez *et al*., 2023; Jauslin *et al*., 2021). Killing mechanisms vary with prey (Jauslin *et al*., 2021; Nasser *et al*., 2013) and the detection of folate is known to play an important role in intracellular killing (Bodinier *et al*., 2020; Leiba

et al., 2017). Whether other chiral signals also regulate intracellular killing is unknown. Given that lytic enzymes are unlikely to harm mirror bacteria, it appears that intracellular killing could be greatly impaired, but whether this would allow mirror *E. coli*, for example, to survive ingestions by *D. discoideum* is unclear.

In summary, *D. discoideum* would likely ingest mirror bacteria at lower rates than natural-chirality bacteria, and it is unclear how effectively mirror bacteria would be killed in the phagosome. After a few hours it is likely that either the mirror bacterium or its largely undigested remains would be egested from the cell, with the protist deriving few, if any, nutrients from it.

natural bacteria, including species which are not typically intracellular parasites (Barker *et al*., 1999). Mirror metabolites released following the successful lysis of a mirror bacterium might also be imported into the protist cytosol or (if hydrophobic) incorporated into the protist lipid membrane, where they might accumulate and may or may not prove harmful to the protist.

To summarize, the mechanisms involved in protist predation are complex and not well understood. While protist species could exhibit diverse responses to a mirror bacterium, in most cases it is likely that key mechanisms would fail to function effectively, resulting in reduced rates of ingestion, killing, and digestion (Figure 8.1).

The nematode *Caenorhabditis elegans* illustrates some of the interactions between bacterivorous animals and mirror bacteria. As discussed in Chapter 6, *C. elegans* is a filter feeder that would ingest mirror bacteria, though the absence of chiral signals may somewhat reduce intake compared to the nematode's preferred prey. Lytic enzymes play key roles in both killing and digesting prey, both on their own and in synergy with other mechanisms such as pharyngeal grinding, which crushes most ingested bacteria (Seymour *et al*., 1983) but is impaired in the absence of lysozyme (Gravato-Nobre *et al*., 2016). Because mirror bacteria are expected to be resistant to these enzymes, it is plausible that those which survive ingestion would be expelled along with other waste. If able to persist within the intestine they could plausibly cause a lethal infection. If infection does not occur, the nematode might learn to avoid mirror bacteria as low-quality food items.

Bacteria are subject to many other forms of predation, including phages (discussed in the previous section), parasitic and predatory bacteria, and a diverse array of animals. A detailed analysis of all possibilities is beyond the scope of this report. Nevertheless, failures of recognition, killing, and digestion are expected to be common. Lytic enzymes, in particular, are used by almost all bacterial predators to kill and digest bacteria (Karasov & Douglas, 2013; Lancaster *et al*., 2019; J. Pérez *et al*., 2016), and while this is usually not the only killing mechanism, it often acts synergistically with others. This deficiency could partially, in some cases perhaps substantially, reduce the mortality rates experienced by mirror bacteria, increasing environmental persistence and potentially allowing net positive population growth in some environments.

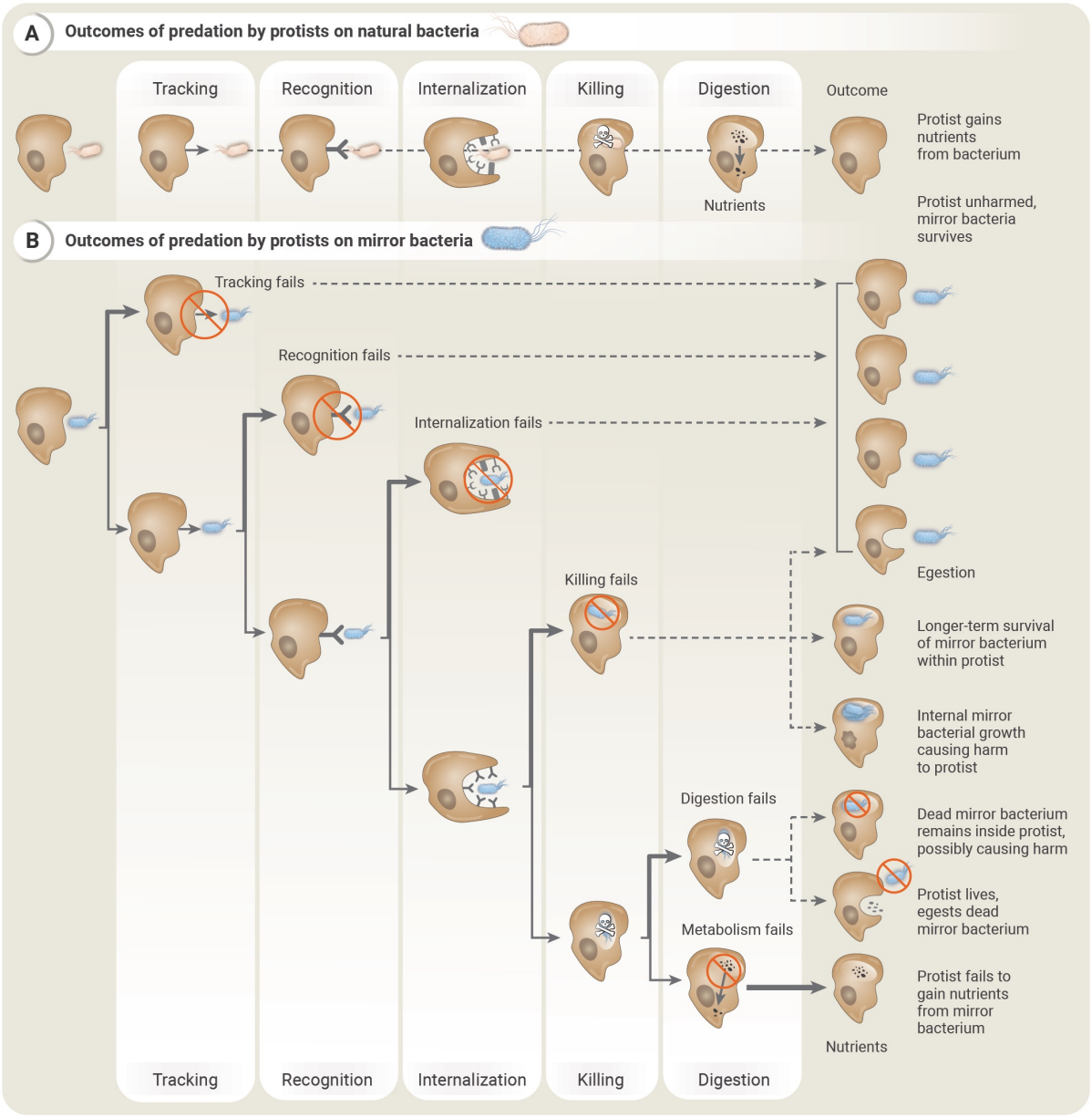

Figure 8.1: Mirror bacteria could be resistant to protists, a major cause of bacterial mortality
(A) Phagocytosis of natural-chirality bacteria by protists relies primarily on chiral mechanisms at each step: tracking, recognition, internalization, killing, and digestion. (B) Phagocytosis of mirror bacteria could fail because each step at least partially depends on the interactions of chiral molecules. Failure of any of the first three steps (tracking, recognition, or internalization) could result in failed predation. Should phagocytosis succeed but the mirror bacteria survive, multiple outcomes are possible, including ones in which the protist suffers harm. Even if mirror bacterial killing occurs, the protist would not be able to properly digest and extract nutrients.

Even predators that do successfully ingest and kill mirror bacteria would derive few nutrients from them. Natural enzymes would fail to decompose mirror proteins and other chiral macromolecules, which make up over 95% of the dry biomass of a typical bacterial cell (Milo & Phillips, 2015). Most small molecule metabolites in bacteria are also chiral (Bennett *et al.*, 2009), and so they too could probably not be catabolized. Under normal conditions, a growing bacterial population facilitates the

Group	Examples	Target / Mechanism of action	Activity vs Mirror	References
Achiral antibiotics				
Dithiolopyrrolones	Holomycin, Thiolutin	RNA polymerase	Yes*	B. Li *et al*., 2014
Nitroimidazoles	Azomycin	Ribonucleotide reductase	Yes*	Saeki *et al*., 1974
Pigments	Prodigiosin, Pyocyanin, Violacein	Various	Yes*	Choi *et al*., 2015; Danevčič *et al*., 2016; Noto *et al*., 2017
Membrane-disrupting antibiotics				
Enniatins	Enniatin B	Lipid membranes	Yes*	Kamyar *et al*., 2004; Shenyakin *et al*., 1967
Lipopeptides	Daptomycin	Phosphatidylglycerol	No*	Moreira & Taylor, 2022
	Surfactin	Lipid membranes	Yes*	Zhen *et al*., 2023
Polyethers	Routiennocin	Ionophores	Yes*	Borcher Møller *et al*., 2024
	Monensin, Salinomycin	Ionophores	Yes*	Rutkowski & Brzezinski, 2013
Polypeptides	Bacitracin A	Bactoprenylpyrophosphate	Yes*	McDougal & Griffin, 2003
	Gramicidin A	Ionophore	Yes*	Providence *et al*., 1995
	Polymyxin B	Lipid A	No*	Slingerland *et al*., 2022

Table 8.1: Naturally occurring antibiotics that could retain efficacy against mirror bacteria
Most antibiotics are unlikely to retain bactericidal activity against mirror bacteria (see Section 5.1). Those listed in this table are exceptions, either because they are achiral or, although chiral, act by disrupting membrane function. For some of the chiral antibiotics, the efficacy of the enantiomer against bacteria has been tested, allowing the efficacy of the natural-chirality antibiotic against a mirror bacterium to be inferred. In those cases marked by *, no such results are available, so the likely efficacy is inferred from what is known about the mechanism of action.

expansion of predator populations, which thereby increases the bacterial mortality rate and keeps the prey population in check (Kirchman, 2018; Levin *et al*., 1977). A growing mirror bacterial population, however, would not support the growth of predators, and so it would likely expand until eventually constrained by resources.

Mirror bacteria would be resistant to many natural antimicrobial toxins

Microbes have evolved a diverse array of toxins and other molecular weapons to harm and kill competitors (Granato *et al*., 2019). Bacterial weapons are particularly well-documented in the gut microbiota of mammals (García-Bayona & Comstock, 2018) and the rhizosphere of plant roots (Doornbos *et al*., 2012; Hassani *et al*., 2018). Almost all known microbial weapons operate by chiral mechanisms and are unlikely to affect mirror bacteria.

Most antibiotics are chiral and typically function by inhibiting chiral enzymes and ribosomes required for key cellular processes such as translation or cell wall biosynthesis (Kohanski *et al*., 2010). Thus, they are unlikely to be effective against a mirror bacterium (see Section 5.1). The only exceptions are those antibiotics that are achiral, act as ionophores, or otherwise disrupt membrane

	Examples	Mechanisms	Target / Receptor	Activity vs Mirror	References
Class I: Modified bacteriocins					
Lantibiotics	Mersacidin	Membrane disruption	Lipid II	No	Hsu *et al*., 2003
	Nisin, Subtilin	Cell wall inhibition, membrane disruption	Lipid II	No	Hsu *et al*., 2004; Parisot *et al*., 2008
Lasso peptides	Capistruin, Microcin J25	Block transcription	RNA Pol III	No	Bayro *et al*., 2003; Kuznedelov *et al*., 2011
Thiopeptides	Thiostrepton	Block translation	50S ribosome	No	Harms *et al*., 2008
	GE2270A	Block translation	EF-Tu	No	Parmeggiani *et al*., 2006
Class IIa: Pediocin-like					
Pediocin-like	Pediocin, **Leucocin A**	Membrane disruption	Mannose-PTS	No	Gravesen *et al*., 2002; Yan *et al*., 2000; Zhu *et al*., 2022
Class IIc: Cyclic peptides					
Circular bacteriocins	AS-48, Carnocyclin, Gassericin	Membrane disruption	No receptor	Yes	Gálvez *et al*., 1991; Gong *et al*., 2009; Kawai *et al*., 2004
	Garvicin ML	Membrane disruption	Maltose transporter	No	Gabrielsen *et al*., 2012
Class IId: Miscellaneous unmodified bacteriocins					
Saposin-like leaderless peptides	**Aureocin A53, Lacticin Q,** Enterocin L50	Membrane disruption	No receptor	Yes	Lander *et al*., 2023; Netz, 2002; Tymoszewska *et al*., 2021; Yoneyama *et al*., 2009
Other	Garvicin Q	Membrane disruption	Mannose-PTS	No	Tymoszewska *et al*., 2017

Table 8.2: Efficacy of broad-spectrum bacteriocins against mirror bacteria
Examples of bacteriocins known to exhibit a relatively broad spectrum of antibacterial activity, listed according to a proposed classification system (Cotter *et al*., 2013). Of these, only certain circular and leaderless bacteriocins act by disrupting membranes without a specific target on the cell surface and so are expected to remain harmful to mirror bacteria. For those examples in **bold**, testing of the enantiomer for antibacterial activity has been reported in the literature.

function (Table 8.1). There are very few naturally occurring achiral antibiotics—all of those on the WHO List of Medically Important Antimicrobials, for instance, are synthetic (World Health Organization, 2024)—but a number of non-ribosomal peptides and polyether ionophores would retain efficacy, primarily against the mirror versions of Gram-positive bacteria.

Bacteriocins are small ribosomally synthesized peptides with diverse mechanisms of action which are usually chiral (Table 8.2). Similar to antimicrobial peptides produced by humans (discussed in Section 4.2) and other eukaryotes, many bacteriocins form pores within membranes or otherwise disrupt membrane function (Acedo *et al*., 2018). Although the basal mechanism is typically insensitive to membrane chirality, bacteriocins normally target the membrane through specific chiral receptors on the cell surface, and consequently are unlikely to retain efficacy against mirror bacteria. This inference has been verified experimentally in the case of leucocin A: its D-enantiomer showed

no antimicrobial activity at the minimum inhibitory concentration of the natural-chirality compound (Yan *et al*., 2000).

The only bacteriocins likely to retain efficacy against mirror bacteria are those that do not require receptors to target membranes. To our knowledge, all such bacteriocins are either circular or leaderless peptides. They have shared structural features and broad-spectrum activity against Gram-positive bacteria, but are only effective against Gram-negative bacteria if the outer membrane is disrupted (Towle & Vederas, 2017). The D-enantiomers of the leaderless peptides aureocin A53 and lacticin Q were recently tested and found to have similar antimicrobial activity to their L-enantiomers (Lander *et al*., 2023), confirming that the natural L-enantiomers should be effective against mirror Gram-positive bacteria.

Larger bacterial toxins, mechanical weapons, and prophages are almost always narrow-spectrum and generally require binding to specific receptors to properly function (Table 8.3). The type VI secretion system provides an exception (Acedo *et al*., 2018), but only when equipped with a pore-forming protein such as Ssp6 or VasX. Because most bacterial weapons have not yet been characterized, additional achiral antibiotics, pore-forming peptides, and perhaps more sophisticated weapon systems that would retain efficacy may exist. Nevertheless, the available evidence suggests that mirror bacteria would be intrinsically resistant to the vast majority of weapons used for bacteria-on-bacteria warfare.

Unlike the specialized weapons considered so far, some metabolic byproducts such as ethanol (Ingram, 1990) and small organic acids (Pinhal *et al*., 2019) act as broad inhibitors of bacterial growth when present at high concentrations. These metabolites are achiral or cause harm through general mechanisms, such as membrane uncoupling, that are not sensitive to chirality. More generally, microbial metabolic processes can create unfavorable growth environments, such as by modifying environmental pH or redox conditions in ways that could prevent a mirror cell that otherwise might colonize from doing so. Therefore, while many forms of interference-based competition between natural microbes and mirror bacteria would likely be impaired, some others could continue to function.

8.2 Mirror bacteria could colonize natural environments outside of multicellular hosts

No matter how predation-resistant they may be, organisms can only colonize new environments if capable of acquiring nutrients sufficient to replicate. Given that many generalist bacteria such as *E. coli* and *B. subtilis* can grow under diverse conditions and on achiral nutrients, there is no fundamental obstacle preventing mirrored versions of such organisms from surviving and replicating in the environment, though low nutrient concentrations or hostile abiotic conditions could create substantial barriers.

An introduced species can colonize an environment if it has a positive net growth rate. Population expansion will continue until one or more ecological feedbacks, such as decreases in nutrient concentrations or increases in predator populations, cause the growth rate to decline. If the

Mechanism	Examples	Target	Activity vs Mirror	References
Bacteriolysins				
Cleaves peptidoglycan (Acedo *et al*., 2018)	Enterolysin A	Peptidoglycan	No	Beukes *et al*., 2000
	Millericin B	Peptidoglycan	No	Khan *et al*., 2013
Pore-forming toxins				
Receptor-dependent pore formation in membranes (Dal Peraro & van der Goot, 2016)	Colicin Ia	Colicin I receptor	No	Buchanan *et al*., 2007
	Pyocin S5	TonB transporter	No	Behrens *et al*., 2020
Prophage				
Infection and lysis (X.-Y. Li *et al*., 2017)	Phage lambda	LamB protein	No	Wendling *et al*., 2021
Tailocin				
Membrane disruption through binding to chiral receptors	R-type pyocins	Chiral receptors	No	Ghequire & De Mot, 2015; Woudstra *et al*., 2024
	F-type pyocins	Chiral receptors	No	
Toxin delivery via CDI				
Toxin delivered via receptor-binding protein syringe (Ruhe *et al*., 2017)	Cdi_STEC031	Tsx	No	Ruhe *et al*., 2017
	EC536	OmpC	No	Ruhe *et al*., 2017
	EC93	BamA	No	Aoki *et al*., 2010
Toxin delivery T6SS				
Toxin delivered by protein syringe that does not require a specific receptor (Galán & Waksman, 2018)	ART-ARHs	FtsZ	No	Ting *et al*., 2018
	RhsA	DNA	No	Koskiniemi *et al*., 2013
	Tle	Phospholipids	No	Russell *et al*., 2013
	Tne2	NAD(P)	No	J. Y. Tang *et al*., 2018
	Tre1	Peptidoglycan	No	Ting *et al*., 2018
	WapA	RNA	No	Koskiniemi *et al*., 2013
	Ssp6	Lipid membranes	Yes	Mariano *et al*., 2019
	VasX	Lipid membranes	Yes	Miyata *et al*., 2013

Table 8.3: Other examples of bacterial weapon systems
Bacteriolysins, pore-forming toxins, and tailocins act on chiral targets on the cell surface, while prophages and toxin-delivery via CDI requires chiral interactions at the cell surface in order to inject effectors into the cell. Only the delivery of lipid-membrane disrupting pore-forming proteins through a T6SS could plausibly retain efficacy against mirror bacteria.

introduced population is initially small or localized, it may suffer extinction through stochastic effects (Dallas *et al*., 2021). However, so long as the expected growth rate is positive, then colonization will occur with some non-zero probability, and with repeated reintroductions successful colonization becomes increasingly likely.

The intrinsic resistance of mirror bacteria to many forms of predation could enable colonization of some environments despite their growth likely being slower than native bacteria. Even environments that cannot be stably colonized by mirror bacteria could act as temporary reservoirs, facilitating

spread between hosts or colonization of other environments, or provide time for adaptations that could allow persistence (Dennehy *et al*., 2006).

Mirror bacteria could obtain nutrients from the environment

All bacteria require nutrients to grow. Cyanobacteria and other photoautotrophs typically require only inorganic nutrients such as nitrogen, phosphorus, and sulfur, which are present in the environment as achiral ions such as ammonia, nitrate, phosphate, and sulfate. *Prochlorococcus*, *Synechococcus*, and *Synechocystis* strains can grow rapidly in fully synthetic media that contain no chiral compounds (L. R. Moore *et al*., 2007; Yu *et al*., 2015). Though some cyanobacteria opportunistically consume chiral organic compounds (Muñoz-Marín *et al*., 2020) and some cyanobacterial strains have chiral vitamin requirements (A. A. Pérez *et al*., 2016), mirror photoautotrophs could likely grow at similar rates to natural-chirality photoautotrophs.

Autotrophs generally do not require any chiral nutrients or chiral interactions with other living organisms to survive. Heterotrophs, by contrast, consume a wide range of low-molecular-weight organic compounds produced by other living organisms or by the breakdown of decaying biomass. Most of these organic compounds are chiral. Concentrations of these nutrients are typically low due to fierce microbial competition, but they suffice to support the slow growth of a diverse array of heterotrophic bacteria (Kirchman, 2016; Rousk & Bååth, 2011).

A mirror heterotroph would likely not be capable of utilizing most chiral nutrients in the environment. The catabolic capabilities of commonly studied heterotrophic bacteria are nevertheless diverse, and this breadth could allow their mirror counterparts to consume a range of common achiral and chiral compounds. For example, a mirrored version of *E. coli* K-12—the most commonly studied bacterial strain in microbiology and biotechnology—could grow on diverse achiral central metabolites, fatty acids, and fermentation products (see Table 1.1). It could also utilize a number of chiral organic compounds, including both enantiomers of alanine, cysteine, serine, xylose, lactate, malate, and tartrate (Chapter 1). These metabolites are often among the most abundant small organic compounds present in many environments. While a mirror *E. coli* would not be capable of catabolizing simple sugars such as D-glucose, it could be engineered to do so by encoding known metabolic pathways (see Table 1.3), which would expand the range of environments that could support mirror bacterial growth and substantially increase their population densities.

Environments are often highly heterogeneous, and many bacteria have evolved to detect and move toward nutrients through chemotaxis (Keegstra *et al*., 2022). Chemotactic receptors are highly promiscuous, binding to a very wide array of biomolecules that might be catabolized. While many of the compounds recognized are chiral, it appears likely that mirrored versions of chemotactic bacteria could slowly move toward nutrients even absent modifications of their receptors. *E. coli*, for example, is attracted to diverse sugars and amino acids including both enantiomers of galactose, glucose, mannose, and xylose (Adler *et al*., 1973), both enantiomers of aspartate and serine, and the achiral molecules ammonium, nitrate, fumarate, and succinate (Mesibov & Adler, 1972; Taylor *et al*., 1979). While affinities for the enantiomers of common sugars are markedly lower, they do suffice for chemotaxis. It therefore appears likely that mirror *E. coli* cells would be able to recognize and find

chiral nutrients, and though they would not have an optimal foraging strategy, they could improve rapidly by natural selection (see Section 8.4). Other mirror bacteria would likely exhibit similar behaviors. Overall, mirror heterotrophs would presumably grow more slowly than native competitors, but some growth appears possible in many cases. And with little or no predation, even slow growth may allow mirror heterotrophs to colonize diverse environments.

The minimal carbon requirements for bacterial growth are very low. For instance, *E. coli*, a bacterium whose primary niche is within the nutrient-rich intestines of warm-blooded animals, can maintain a positive growth rate of ~0.2 hr^{-1} with only 20 µg/L glucose as its sole carbon source (Füchslin *et al*., 2012; Shehata & Marr, 1971). While estimates of minimal required concentrations for other nutrients are not available, it is plausible that *E. coli* can slowly grow on a wide variety of substrates—including those in Table 1.1—at concentrations in the hundreds of micrograms per liter, and thus potentially proliferate due to their intrinsic resistance to predation.

In natural waters, many nutrients that could be accessible to mirror bacteria are typically present at concentrations of 1–1000 µg/L (Thurman, 2014), including simple organic acids such as acetic, butyric, glycolic, lactic, and propionic acids; amino acids such as L-alanine and L-serine; and D-glucose, D-fructose, and other common sugars that could be available to a suitably engineered mirror bacterium. Though organic carbon might be too sparse to support mirror *E. coli* growth in seawater, this may not be true of all mirror heterotrophs. Moreover, growth in some rivers, eutrophic lakes, and marshes could be possible for many kinds of mirror heterotrophs.

Soils also contain diverse organic compounds. Sugars are the most important carbon and energy source for soil microorganisms (Gunina & Kuzyakov, 2015), while organic acids including acetic, fumaric, glycolic, lactic, and malic acid are also common due to exudation by plant roots (Kamilova *et al*., 2006) and release by microbes (Adeleke *et al*., 2017). Free amino acids are also significant contributors to soil organic matter (Hu *et al*., 2018; Senwo & Tabatabai, 1998; Werdin-Pfisterer *et al*., 2009), and D-amino acids are usually present, though at lower concentrations (Vranova *et al*., 2012). While abiotic factors can limit the accessibility of these nutrients—particularly water activity (R. F. Harris, 1981) and redox potential (Husson, 2013)—some soils could plausibly support growth of a mirror *E. coli* strain or similar mirror heterotrophic bacteria, especially one engineered to metabolize D-glucose and other common sugars.

Bacterial growth in soils is typically limited by carbon (Demoling *et al*., 2007), but phosphate and nitrogen concentrations can be limiting in natural waters (Paerl, 2018; Sterner, 2008). Marine phosphate concentrations, for instance, are typically much lower than 1 µM (Paytan & McLaughlin, 2007), which is far too low to support *E. coli* growth (Shehata & Marr, 1971). In most environments, however, mirror heterotroph growth would probably not be prevented by a lack of inorganic nutrients.

Iron is an interesting case. The Fe^{3+} ion is highly insoluble, and so in oxygenic environments iron is almost exclusively found complexed with organic molecules. A common strategy bacteria use to acquire iron is to produce siderophores, which are small iron-binding molecules released into the environment and subsequently reabsorbed. These siderophores are highly diverse and usually, though not always, chiral (Sandy & Butler, 2009). Mirror bacteria are therefore unlikely to be able to use

naturally occurring siderophores, and likewise natural-chirality bacteria could probably not use the siderophores produced by mirror bacteria. Because siderophores are released into the environment, they can act as "public goods" benefiting all strains able to utilize them, including "cheaters" that consume but do not produce siderophores (Kramer *et al*., 2020). The extent to which producing and consuming reversed-chirality siderophores would prove harmful, or perhaps even beneficial, to mirror bacteria is unclear.

In the specific case of *E. coli*, it is known that mirror ferric enterobactin, the most commonly utilized siderophore, is imported into the cytoplasm but not cleaved, and therefore cannot be used to obtain iron (Abergel *et al*., 2009). A mirror *E. coli* would produce mirror enterobactin, but would be unable to utilize natural-chirality enterobactin; thus, natural-chirality and mirror *E. coli* would likely interfere with each other's ability to acquire iron through enterobactin. *E. coli* can also obtain iron from iron(III)-citrate complexes and, in anoxic environments, by directly importing Fe^{2+} (Braun, 2003); these iron acquisition mechanisms would not be sensitive to chirality.

Mirrored bacteria could be robust to abiotic stressors

In addition to nutrients, bacterial growth also requires tolerable environmental conditions. Abiotic stressors such as temperature, pH, oxidative stress, and osmotic pressure are achiral, and organismal responses to these stressors seldom involve chiral interactions with the external environment. A mirror bacterium would therefore have similar tolerances to abiotic stressors as its natural-chirality equivalent.

The first mirror bacteria, if created, might be fragile and functionally inflexible, akin to existing highly laboratory-adapted or engineered cells (Fredens *et al*., 2019; Hutchison *et al*., 2016). As described in Chapter 3, however, transforming these initial fragile cells into analogs of more robust bacterial species through gene addition would be feasible and required for most applications. A mirror *E. coli*, for instance, would be a natural target given the central role played by this model organism in both research and biotechnological applications. While the primary niche of *E. coli* is the mammalian gut, it can survive and replicate under diverse environmental conditions (Box 8.2).

We have focused on *E. coli* to this point because, as a model organism, a great deal is known about its resource utilization and environmental tolerances. Importantly, some bacterial species are more robust than *E. coli* in many environments. A mirror version of an environmental generalist such as *Pseudomonas aeruginosa* would likely exhibit better survival in many soils and natural waters. A mirror version of *Vibrio* would likely thrive in seawater if able to acquire nutrients, and a mirror cyanobacterium such as *Prochlorococcus* or *Synechococcus* would not even require organic carbon to grow.

While abiotic stressors are themselves achiral, some responses can involve chiral mechanisms that may be altered or impaired in mirror bacteria. For example, during osmotic stress *E. coli* preferentially imports L-proline as a compatible solute and cannot similarly use D-proline (Sasaki *et al*., 2007). A mirror *E. coli* could not import L-proline, though it could still import the achiral betaine (Cayley *et al*., 1992), and in the absence of either preferred osmolyte could simply import potassium ions.

Box 8.2: *Escherichia coli* can survive and replicate under many environmental conditions

While primarily known as an intestinal commensal of warm-blooded animals, *E. coli* is capable of survival and growth under a wide range of conditions if nutrients are available. Many strains can sustain growth between ~8℃ and as high as ~48℃ (Baka *et al*., 2013; Ross *et al*., 2003), and they readily survive colder temperatures including even freezing (Sleight *et al*., 2006). Strains can grow between pH of ~4 to ~9 (Baka *et al*., 2013; Ross *et al*., 2003), and many can survive more acidic conditions for hours (Gorden & Small, 1993). *E. coli* can even grow in seawater if nutrients are supplemented (Jannasch, 1968).

Such conditions are characteristic of many natural environments, implying that *E. coli* can survive for extended time periods in many soils and natural waters. Studies of pathogenic strain O157:H7, for instance, find that it can survive for many weeks or months in soil (Alegbeleye *et al*., 2018; Mukherjee *et al*., 2006; Ongeng *et al*., 2011; Yao *et al*., 2013; Zhang *et al*., 2013), feces and manure (Fukushima *et al*., 1999; Jiang *et al*., 2002; Kudva *et al*., 1998), and freshwaters (Ogden *et al*., 2001; Wang & Doyle, 1998).

Predators appear to be a major cause of *E. coli* mortality in the environment, which is particularly important considering that a mirror *E. coli* strain would be resistant to many forms of predation. Studies that compare *E. coli* K-12 or O157:H7 growth in untreated vs. pre-sterilized soils, natural freshwaters, and seawater find greatly enhanced survival, and sometimes net growth, in the sterilized samples that lack phages, predators, and competitors (Bogosian *et al*., 1996; W. L. Chao & Feng, 1990; Rozen & Belkin, 2001; Vital *et al*., 2008). *E. coli* K-12 cells can persist for months in sterile soil and natural freshwaters (Bogosian *et al*., 1996; Flint, 1987) and for over a week in sterile seawater (Byrd & Colwell, 1990; Gauthier & Le Rudulier, 1990; Sørensen, 1991). If seawater is enriched with nutrients, *E. coli* can even outcompete marine heterotrophs (Jannasch, 1968).

While neither K-12 nor O157:H7 are generally able to sustain population growth outside of their hosts, naturalized *E. coli* strains have been discovered in diverse environments (Ishii & Sadowsky, 2008; Jang *et al*., 2017; Luo *et al*., 2011), including in various soils (Byappanahalli & Fujioka, 2004; Ishii *et al*., 2006; Solo-Gabriele *et al*., 2000; Topp *et al*., 2003), sands (Beversdorf *et al*., 2007; R. L. Whitman & Nevers, 2003), sediments (Desmarais *et al*., 2002), and natural freshwaters (Byappanahalli *et al*., 2003; Carrill *et al*., 1985). The fact that *E. coli* can become naturalized to such varied environments raises the possibility that a mirror *E. coli*, which would be resistant to most forms of predation, might also be capable of colonizing similar environments.

Biofilm production is another common response to abiotic stressors. Given that many bacteria can attach themselves to abiotic surfaces through often non-specific interactions (Beloin *et al*., 2008; Berne *et al*., 2018), the general ability for mirror bacteria to form biofilms on abiotic surfaces would not be impaired. However, the recognition of and attachment to biological surfaces may be impaired in some cases, as could participation in biofilms produced by natural bacteria. Reversed chirality

might also present some benefits: mirror bacteria would be resistant to many of the antagonistic interactions that are common in dense microbial communities (Rendueles & Ghigo, 2012; Stubbendieck & Straight, 2016), and they would excrete extracellular polymeric substances that would not easily be degraded by naturally occurring enzymes.

In addition to broad physical-chemical conditions such as temperature and osmotic pressure, bacterial growth can also be inhibited by the presence of specific toxic molecules. There is a possibility that toxicities of chiral molecules might pose a barrier to environmental colonization. As discussed in the Introduction, certain chiral compounds such as L-tryptophan or L-cysteine could be toxic to mirror bacteria, but the requisite concentrations, at least for mirror *E. coli*, are much greater than those of almost any organic molecule typically found in natural environments. Given the low concentrations of nutrients in the environment and the lack of evidence that any common metabolites would be particularly toxic to mirror bacteria, it appears unlikely that chiral incompatibilities would prevent growth of mirror bacteria in otherwise hospitable ecosystems.

In summary, a mirror *E. coli* or other generalist bacterium may be capable of abiotic survival in many environments, including most inhabited by humans, and could likely grow in the presence of sufficient nutrients. Some responses to abiotic stressors, such as biofilm production or osmolyte uptake, could be affected by their reversed chirality, but this appears unlikely to substantially reduce fitness in most environments. Moreover, any mirror bacteria that colonized a natural environment would experience evolutionary pressure to adapt to the local conditions, enabling them to grow to higher densities and expand their range. We return to this issue in Section 8.4.

Colonization requires growth to outpace mortality

Even though bacteria can grow in a wide variety of environments, optimal growth is typically restricted to a narrow range of conditions: even bacterial "generalists" are typically comprised of many different strains that thrive in slightly different niches. It might therefore seem unlikely that a single mirror strain could compete successfully with natural bacteria across many different ecosystems. Unfortunately, even setting aside the evolution of the mirror strain (see Section 8.4), this intuition is incomplete, because mirror bacteria would likely evade predation to an unprecedented degree. If mirror bacteria are capable of net positive growth in an environment, then colonization is possible even if their replication rates are low.

While the first robust mirror bacteria are likely to be heterotrophs, such as *E. coli*, it is instructive to first consider the balance between growth and death of a mirror autotroph. *Prochlorococcus* strains are the most abundant cyanobacteria in the ocean, and are found in most tropical and subtropical waters (Flombaum *et al*., 2013). Growth rates for *Prochlorococcus* are typically around 0.5 day^{-1} (Kirchman, 2016), matched, on average, by an equal loss to predation by protists and phages. There are many different ecotypes and strains of *Prochlorococcus*, each of which grows optimally under a limited range of conditions (Biller *et al*., 2015). The *Prochlorococcus* eMIT9312 strain, for instance, grows optimally at 25℃ but can maintain growth between 16℃ and 32℃ (Johnson *et al*., 2006). In natural environments, eMIT9312 will only dominate at higher temperatures, not because it cannot grow at 20℃, but because it is outcompeted by other *Prochlorococcus* strains that are better adapted

to cooler waters and grow faster than eMIT9312 at lower temperatures. At their equilibrium density, these cold-tolerant strains support populations of phages and protists that hold the cold-tolerant strains in check, but which would consume eMIT9312 faster than it can grow.[16] While eMIT9312 may be capable of replicating at 0.4 day^{-1} in cooler waters, it will not persist if its mortality rate is 0.5 day^{-1}.

A mirror eMIT9312 would grow at a similar rate to its natural-chirality counterpart, since it requires only sunlight and achiral inorganic nutrients.[17] But it would fall prey to phages and protists at substantially lower rates, greatly increasing the average lifespan of the mirror strain. While the mirror strain would still grow in 20℃ water at a rate of 0.4 day^{-1}, its death rate would be much lower than that, allowing it to invade areas such as cooler waters that its natural equivalent could not (Figure 8.2). The abiotic limits on growth would still hold—mirror *Prochloroccus* eMIT9312 could not colonize water below 16℃, although it might evolve to do so over time. It would also be unlikely to entirely outcompete native *Prochlorococcus* strains in the regions it colonized (see Section 8.5). Nevertheless, the mirror strain could probably colonize large fractions of the ocean even without evolution. Such a mirror bacterium would properly be considered an invasive species, and the consequences for marine ecosystems and even global biogeochemistry could be severe (Section 8.5).

There are several important differences between a mirror heterotroph and a mirror photoautotroph, but the core colonization dynamics are fundamentally similar. Natural heterotrophs exist in a quasi-steady state where slow grow rates—typically around 0.03–0.3 day^{-1} in both soils (Rousk & Bååth, 2011) and marine environments (Kirchman, 2016)—are balanced by predation and other causes of mortality. A mirror heterotroph would likely have a substantially lower growth rate than natural heterotrophs due, in part, to a limited ability to acquire chiral nutrients. However, the mirror heterotroph would probably also have a much lower mortality rate because it could evade most forms of predation and microbial antagonism. Accessible carbon is often very limited in the environment, and in many cases this might preclude growth, and hence colonization, entirely. But if the mirror heterotroph can grow even slowly, and if its growth rate exceeds its mortality rate, then it would have net positive population growth. Thus, the mirror heterotroph could colonize the ecosystem, despite having slower growth rate than the native heterotrophic bacteria.

As described previously, there does not appear to be any fundamental obstacle to the growth of mirror heterotrophic bacteria in the environment. It is plausible that a mirror *E. coli*, for example, would be capable of growth in some soils and natural waters, particularly if able to catabolize D-glucose and other common sugars. Given that a mirror *E. coli* would likely be resistant to most forms of predation and microbial weapons, this advantage may allow it to colonize some environments even if it has other major disadvantages. Other mirror heterotrophs would differ in

[16] Note that competition between *Prochlorococcus* strains is not perfect. Any given region of the ocean may contain hundreds of genetically distinct subpopulations (Kashtan *et al*., 2014), though usually one ecotype is predominant (Farrant *et al*., 2016). The mechanisms governing this coexistence are not well understood, though variable resistance to cyanophages is likely an important factor (Biller *et al*., 2015; Rodriguez-Valera *et al*., 2009).
[17] *Prochlorococcus* can opportunistically consume chiral organic compounds (Muñoz-Marín *et al*., 2020), so there could be some disadvantage, but in practice this would probably be small.

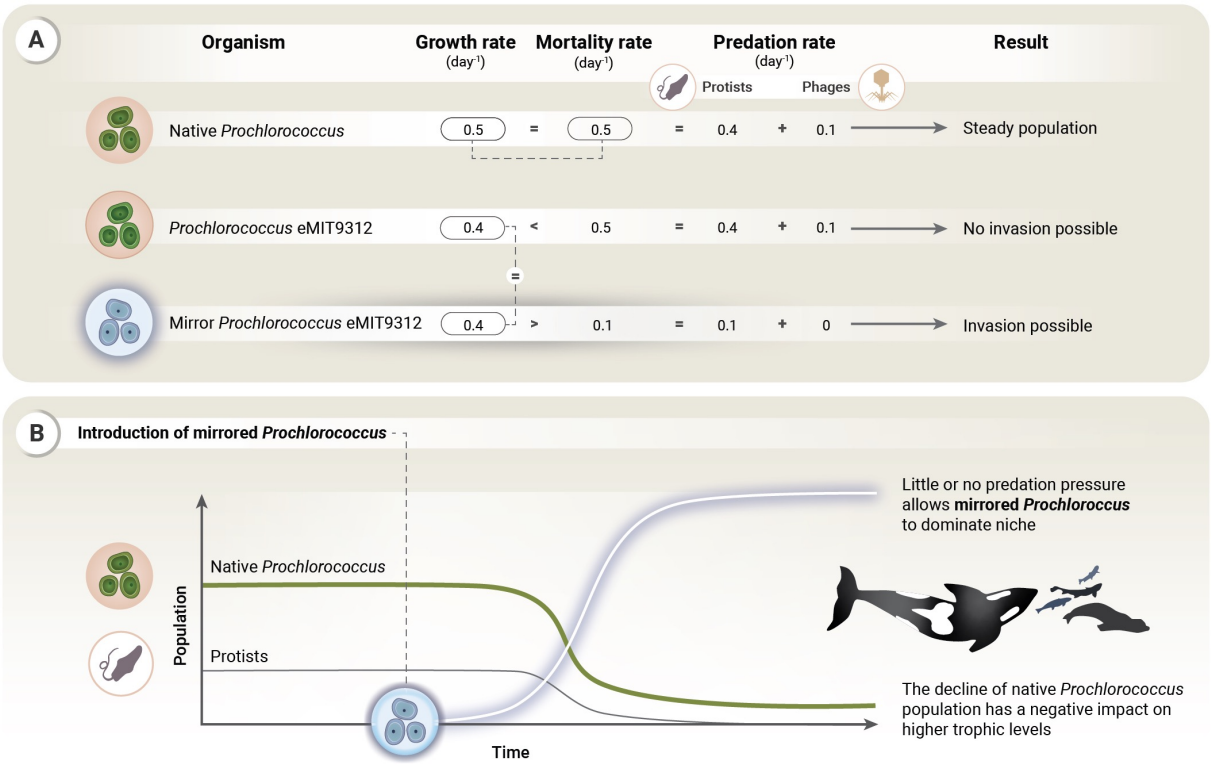

Figure 8.2: Mirror phototrophic bacteria could evade predation, thereby colonizing large fractions of the ocean with severe consequences for marine ecosystems
A. Illustrative population growth and mortality rates of various *Prochlorococcus* strains show the potential consequences of mirror *Prochlorococcus* invasion. The precise rates would vary in real-world scenarios, but the values here are roughly in line with available data (Kirchman, 2016), though predation rates (including the split between protists and phages) are more uncertain. In this scenario, a native *Prochlorococcus* strain exhibits a growth rate (0.5 day^{-1}) roughly matching predation rates by protists and phages (0.4 day^{-1} and 0.1 day^{-1}, respectively, for a total of 0.5 day^{-1}), thereby resulting in a steady population. Non-native *Prochlorococcus* strains that are maladapted for this environment, here the eMIT9312 strain, will have slightly lower growth rates (0.4 day^{-1}) but similar predation rates (0.5 day^{-1}), and therefore cannot survive in conditions better suited to the native strain. A mirror eMIT9312 strain could exhibit similar growth rates to its natural-chirality, non-native counterpart but experience significantly decreased predation rates, speculatively shown here as a total of 0.1 day^{-1}, thereby allowing invasive growth. **B.** Invasive growth could allow mirror *Prochlorococcus* strains to dominate niches normally occupied by multiple natural *Prochlorococcus* strains, thereby causing a decline in natural *Prochlorococcus* populations and the protists that feed on them. The resulting reduction in digestible biomass could drive many animal species that are higher in the food web to extinction (see Section 8.5).

their catabolic capabilities and robustness to abiotic stressors but could create similar, and in some cases even greater, concerns.

Mirror bacteria capable of colonization could become highly abundant

Even at a modest net growth rate of 0.1 day^{-1}, a single bacterium unchecked by nutrient limitations or predation could give rise to roughly 5×10^{31} descendants in just 2 years—more than the estimated total number of bacteria on the planet (Bar-On *et al*., 2018; W. B. Whitman *et al*., 1998). Of course, no population can grow forever. As populations grow, they deplete nutrient supplies and other

resources ("bottom-up control"), and they become prey to a growing population of predators and parasites ("top-down control"), eventually reducing the net growth rate and causing the population to stabilize or decline.

We have already discussed both nutrients and predation in the context of initial colonization—namely, could a small initial population of mirror bacteria sustain net positive growth? Here, the issue is slightly different—how would nutrient availability and predation pressure change as the mirror population grows? Reduced predation and parasitism is a common feature of many invasive species (the "enemy release hypothesis"; Keane & Crawley, 2002). Nevertheless, at least some predation and parasitism usually occurs, and the corresponding predator and parasite populations expand in response to growth of the invasive species.

As discussed in Section 8.1, a similar response to colonizing mirror bacteria is unlikely. Mirror bacteria would provide few if any nutrients to predators, precluding any commensurate expansion in predator or parasite populations as the mirror bacteria proliferate. Mirror bacterial populations would thus escape the normal mechanism of top-down control, allowing them to grow unchecked until limited by the available nutrients. Reduced predation would reduce the overall mortality rate and lower the minimum nutrient concentration necessary to sustain an equilibrium population, which in simple ecological models is what ultimately determines the winner in resource competition (Tilman, 1982). Mirror bacteria would essentially be accidental "defense-specialists" (Våge *et al*., 2014; Winter *et al*., 2010) with very low mortality due to predation, and this could allow them to reach population densities much larger than those typically achieved by individual bacterial strains in nature. The effect could be particularly strong in environments where bacterial mortality is dominated by predators. By contrast, mirror bacteria may struggle to colonize or outcompete native bacteria in environments where abiotic processes are the primary driver of bacterial mortality, as the advantages of reversed-chirality would be relatively small. Section 8.5 discusses potentially severe and irreversible harms that could result from mirror bacterial colonization and growth in natural ecosystems.

Environmental reservoirs would facilitate the spread of infectious mirror bacteria

As discussed in Chapters 4, 6, and 7, mirror bacteria appear likely to enter animals and plants through a variety of mechanisms and may establish infections due to their anticipated immune evasion. Because mirror bacterial translocation through the animal gut appears plausible (see Sections 4.4 and 6.1–2), predators, parasites, and scavengers could be infected as well, then spread the mirror bacterium to other organisms through the food web. With many potential hosts and the presumed capacity to spread between them, mirror bacteria could potentially persist in some ecosystems even if they were largely incapable of surviving in the environment. The kinetics of infection and population expansion within each host species would be important for the rate of geographic spread (see Section 8.4), but any mirror bacterium capable of reaching a dense population within a host organism could plausibly spread to others that feed upon this host.

Infected animals would repeatedly introduce mirror bacteria into soils and natural waters. When the animal dies, it could release a relatively large population of mirror bacteria into the environment,

reducing the likelihood of local extinction. Any external environment susceptible to colonization by mirror bacteria might eventually be colonized, creating a permanent reservoir of mirror bacteria that could in turn infect new multicellular hosts.

Some environments could not be stably colonized by any given mirror bacterium. There might simply be too few nutrients available to support mirror bacterial growth, the abiotic conditions might not be suitable, or acute abiotic stresses might cause mortality that outpaces growth. Environments with low rates of bacterial mortality from predators or microbial warfare may be particularly unlikely to be colonized, as the ecological advantages of reverse chirality result from disruptions to these processes. Even so, such environments could act as temporary reservoirs for mirror bacteria. Persistent environmental phases are common for many bacterial commensals and pathogens. *E. coli* can persist for months in soils and natural waters (Box 8.2), which is unsurprising given its biphasic life cycle (Blount, 2015), and pathogens such as *Bacillus anthracis* (Hugh-Jones & Blackburn, 2009) and *Vibrio cholerae* (Faruque *et al*., 1998) also persist for extended periods in the external environment between host infections.

Mirror bacteria could be made similarly robust to abiotic stressors while also avoiding predation and other biological processes that commonly remove pathogens from the environment. They could also potentially infect a greater range of animal and plant hosts. Mirror bacteria might therefore reach relatively high densities even in environments that they cannot colonize, as they would be repeatedly reintroduced by dying infected organisms. The presence of a large transient mirror bacterial population in soils and natural waters could in turn accelerate the evolution of adaptations that would facilitate true colonization of these environments (Kawecki, 2008).

8.3 Invasive mirror bacteria could rapidly disperse through the environment

A population of mobile organisms with a net positive growth rate can expand until it reaches the boundaries of the hospitable region. In the case of mirror bacteria, we expect that most movement would occur via multicellular host organisms or human transport. Mirror bacteria could plausibly infect and be carried by many different animal hosts, and they would presumably be as able as natural bacteria to hitchhike on vehicles. Therefore, the rates of spread of historical invasive species can provide an indication of how rapidly they would disperse.

Invasive species have been reported to spread at rates ranging from 1–100 km/yr for invasive mammals and insects (Shigesada & Kawasaki, 2001) to 50–10 000 km/yr for plant and animal pathogens (Kerr, 2012; McCallum *et al*., 2003) (Table 8.4). Pandemic human viruses are the fastest of all: SARS-CoV-2 spread to every inhabited continent within four months of the first case (Ali *et al*., 2020).

Invasive species	Origin	Spread rate (km/year)	References
Animals			
Earthworm, *Lumbricus rubellus*	Netherlands (20th C)	0.014	Marinissen & van den Bosch, 1992
Sea otter, *Enhydra lutis*	California (20th C)	1	Lubina & Levin, 1988
Muskrat, *Ondatra zibethica*	Czechoslovakia (1905)	13	Andow *et al*., 1990
Cereal leaf beetle, *Oulema melanopus*	Michigan (1958)	58	Andow *et al*., 1990
Rabbit, *Oryctolagus cuniculus*	Australia (19th C)	100	Alves *et al*., 2022
Pathogens			
Dutch elm disease, *Ophiostoma ulmi*	Britain (1960s)	50	Harwood *et al*., 2011
Rabies, *Rabies lyssavirus*	Europe (20th C)	50	Anderson *et al*., 1981
Mycoplasmal conjunctivitis, *Mycoplasma gallisepticum*	USA (1994)	510	Dhondt *et al*., 1998
Coral bacterium, *Sphingomonas*	Florida (1995)	600	Richardson *et al*., 1998
Zika virus, ZKV	Brazil (2015)	2190	Lowe *et al*., 2018
Myxomatosis virus, *Myxoma*	Australia (1950)	7300	Kerr, 2012
Severe acute respiratory syndrome virus, SARS-CoV-2	World (2020)	48000	Ali *et al*., 2020

Table 8.4: Rates of spread of animals and novel pathogens during historical invasions and epidemics

Animal hosts could spread invasive mirror bacteria throughout the environment

The spread rate of an animal pathogen depends on how rapidly new hosts become infected and how far they travel before transmission. The simplest diffusion models of biological invasions predict a constant velocity of spread (Lewis *et al*., 2016; J. D. Murray, 2003):

$$v = 2\sqrt{D\lambda}$$

where λ is the growth rate of the infected population and D is the diffusion rate for the movement of infected individuals. These models give reasonable estimates for the spread rate of many invasive species and pathogens (Andow *et al*., 1990; J. D. Murray *et al*., 1986; Noble, 1974; van den Bosch *et al*., 1992). In some cases, animal movements include small probabilities of long-distance movements ("jump dispersals"), which can cause much faster spread velocities (Clark *et al*., 1998; Nathan, 2006; Neubert & Caswell, 2000; Pyšek & Hulme, 2005; Suarez *et al*., 2001), a result readily derived from more general growth-dispersal models (Kot *et al*., 1996; Lewis *et al*., 2016).

A mirror bacterium would be a novel kind of pathogen, but if it were transmitted between animal hosts, it should spread approximately as fast as other diseases transmitted between similar hosts with similar growth rates. Since many different species may be susceptible to infection, the spread of an invasive mirror bacterium would likely be determined by those hosts that travel furthest and transmit most readily.

Flying insects are responsible for some of the fastest spreading terrestrial animal pathogens, including the myxoma and Zika viruses (Table 8.2). However, even mirror bacteria capable of infecting most insects are unlikely to spread as rapidly as these insect-vectored viruses, which tend to replicate faster within hosts than existing pathogenic bacteria. Moreover, mirror strains are unlikely to be efficiently transmitted via mosquito salivary glands, although a few bacteria (*Francisella tularensis*, *B. anthracis*, and *Rickettsia felis*) can be transmitted by mosquitoes via an unknown mechanism (Abdellahoum *et al*., 2020).

Even mirror bacteria that are not very infectious or are limited in their host range could still spread reasonably quickly. Rabies has an incubation period of roughly 1 month (Toma & Andral, 1977) and is spread only by physical contact with an infected animal. As a result, the pathogen spread at a comparatively slow ~50 km/yr (Anderson *et al*., 1981) through European foxes in the 20th century, which was still rapid enough to spread across the continent in a few decades. A mirror bacterium capable of infecting humans would likely also infect other terrestrial vertebrates, possibly including migratory birds, and rabies provides a conservative lower bound for the spread rate in most inhabited regions.

Human activity could spread invasive mirror bacteria globally in just a few months

Modern humans travel much further and faster than any migratory animal; air travel allows an infected person to cross and move between continents within a day. Global travel explains why SARS-CoV-2 spread much faster than any animal virus.

Mirror bacteria could also be transported on contaminated items, within transported animals or plants or products made from them, or perhaps via hitchhiking insects (Gratz *et al*., 2000; Tatem *et al*., 2012). Container ships (Kaluza *et al*., 2010; Lounibos, 2002; Ruiz *et al*., 2000) transport goods across the planet and would also inadvertently spread mirror bacteria, as would trains, trucks, and cars traveling across continents (Ansong & Pickering, 2013; Gippet *et al*., 2019). As noted in the previous section, such human-mediated "jump dispersals" can greatly increase the rate at which invasive species spread. It is difficult to envision any plausible way to avoid the inadvertent spread of an invasive mirror bacterium to most populated locations without ceasing nearly all global travel and cargo transport. Even if such extreme measures were feasible, they would not prevent more localized spread of mirror bacteria through natural animal populations.

Outside of animal hosts or human vehicles, mirror bacteria could still spread through terrestrial environments, albeit more slowly. Bacterial pathogens are often transported by water, including through surface runoff, by rivers and streams, and during flooding events (Boithias *et al*., 2021; Bradford *et al*., 2013; Cho *et al*., 2016); mirror bacteria present in soil could be similarly transported. Bacterial plant pathogens are often dispersed through wind and rain, generally over short distances, but occasionally over several kilometers (West, 2014). Long-distance transport by wind is possible, though infrequent (Mayol *et al*., 2017). Bacterial survival during atmospheric transport is challenging due to mortality from ultraviolet radiation and desiccation (Fernandez *et al*., 2019; J. W. Tang, 2009), but some transport does occur, plausibly facilitated by co-transportation and shielding by dust or dead biomass (Griffin *et al*., 2017; Yoo *et al*., 2019). These and similar jump dispersals may be responsible

for the global mixing of prokaryotic species over many thousands of years (Louca, 2022), though anthropogenic transport likely dominates in the contemporary world.

Marine mirror bacteria could spread through ocean currents

In marine environments the movement of water passively disperses organisms. Effective diffusion rates from turbulent mixing are ~1 km^2/day over a few days (Okubo, 1971; Thorpe, 2012) and 5–50 km^2/day over timescales greater than a couple of weeks (Zhurbas & Oh, 2004). Given a positive growth rate within the water column, an invasive mirror *Prochlorococcus* or other marine bacterium could spread through the ocean at velocities of a few kilometers each day due to turbulent diffusion.

A mirror *E. coli* may not be able to replicate in seawater due to the paucity of nutrients (particularly phosphorus). Nevertheless, *E. coli* can survive in seawater for several days (see Box 8.2). A mirror *E. coli* capable of infecting marine mammals or other marine organisms might therefore spread several kilometers each day by turbulence and currents when outside of hosts, much like the pathogenic coral bacterium *Sphingomonas* spread through Floridian reefs at 2 km/day on ocean currents in 1995 (Richardson *et al*., 1998).

8.4 Invasive mirror bacteria could rapidly evolve and diversify

Natural bacteria evolve when errors in genome replication create mutations, some of which modify traits affecting survival or reproduction. Mirror bacteria would be no different. If invasive mirror bacteria were released into the environment, they could quickly adapt and diversify, further expanding the range of susceptible hosts and ecosystems.

This section focuses on the initial evolution of mirror bacterium subsequent to colonization of an environment. Existing organisms would also evolve in response to mirror bacteria. Evolution in response to mirror bacteria would, however, only occur once the mirror bacteria are sufficiently abundant to apply selective pressure on natural organisms. Such adaptations could have important consequences for the long-term ecological response to mirror bacteria but would have limited relevance during the initial expansion of an invasive mirror bacterium.

Mirror bacterial evolution would depend on new mutations, which could arise quickly

A mirror bacterium that accidentally escapes containment would likely start with essentially no standing genetic variation. Mirror bacteria would be incapable of horizontal gene transfer with normal-chirality organisms, which would prevent the acquisition of novel genes and functions from other organisms. As a consequence, the genetic diversity that fuels adaptation by natural selection would have to be generated through *de novo* mutations arising after release. Even so, evolution that relies entirely on new mutations can fuel rapid and extensive adaptation, especially when bacteria encounter new environments.

Bacterial populations can replicate and expand quickly, with typical point mutation rates of 10^{-10} to 10^{-9} mutations per nucleotide per generation (Lynch *et al*., 2016). The total number of bacteria in a typical human is about 4×10^{13} (Sender *et al*., 2016). While the total number of mirror bacteria that

would arise during an infection is highly uncertain and would in part depend on the size of the infected animal, populations of 10^{10} or more are certainly plausible. Assuming a genome size of several Mb (million bases), nearly every single possible point mutation could be generated and subjected to natural selection in the first large animal to become infected.

As the mirror bacterial population expands, lineages would experience different rates of adaptation due to variability in replication and mutation rates. Replication rates are highly variable because the environment is highly variable: doubling times within a host animal could be measured in hours or even minutes, whereas the strain might require many days to divide in soils and natural waters. Mutation rates can be dramatically increased by mutations to DNA replication and repair mechanisms. Such "mutator" phenotypes typically rise to high frequencies when bacteria encounter new selective conditions (Mao *et al*., 1997; Oliver *et al*., 2000; Sniegowski *et al*., 1997; Taddei *et al*., 1997; Tenaillon *et al*., 2016; Wiser *et al*., 2013), where they can accelerate the process of genetic adaptation. Mirror bacteria that are spreading invasively through new environments appear likely candidates to evolve hypermutability.

Lineages that reach higher population sizes would explore more mutations and thus may evolve more rapidly. These fast-adapting mirror lineages could experience hundreds or even thousands of generations each year, and with huge populations they would continuously generate new mutations. While deleterious changes would generally be eliminated by natural selection, these lineages could accumulate many neutral and, more consequentially, beneficial mutations each year (Tenaillon *et al*., 2016).

Natural selection could rapidly increase the fitness of an invasive mirror bacterium

Laboratory evolution experiments have demonstrated that bacterial populations can rapidly adapt to new conditions. Over the first 2 000 generations (about a year) of the long-term evolution experiment (LTEE), the fitness of the evolving *E. coli* populations increased by more than 20% relative to their ancestors in a benign environment without predators, competitors, or abiotic stresses. Similar studies in which *E. coli* were exposed to more substantial challenges have observed even faster and larger fitness improvements (Baym *et al*., 2016; Tenaillon *et al*., 2012). In the LTEE, the adaptation rate shows strongly diminishing returns, but fitness continues to increase. After 50 000 generations (about 22 years), relative fitness had increased by about 75% in those populations that evolved hypermutability, which accumulated 500–2 500 mutations, and by about 60% for those that retained the lower ancestral mutation rate, which had acquired an average of 60–100 mutations (Tenaillon *et al*., 2016; Wiser *et al*., 2013).

Other experiments have demonstrated that a wide variety of specific adaptations can evolve quickly in *E. coli*, including improved tolerance of acidity (Harden *et al*., 2015), oxidative stress (Rodríguez-Rojas *et al*., 2020), osmotic stress (Guo *et al*., 2017), ionizing radiation (D. R. Harris *et al*., 2009), starvation (Finkel & Kolter, 1999), high temperatures (Rudolph *et al*., 2010), freeze-thaw cycles (Sleight *et al*., 2008), and antibiotics (Baym *et al*., 2016). *E. coli* can rapidly evolve to increase growth rates on non-preferred or even non-native carbon sources (Blount *et al*., 2012; Fong *et al*., 2003; Herring *et al*., 2006; Izutsu & Lenski, 2022; Lee & Palsson, 2010), and they can quickly

overcome low growth rates from metabolic imbalances caused by genome reduction (Choe *et al*., 2019; Hitomi *et al*., 2024; Moger-Reischer *et al*., 2023). Mutagenesis experiments show that mutants of *E. coli* K-12 can utilize numerous non-native nutrient sources (see Table 1.1–3), including the right-handed forms (i.e., reverse chirality) of eight of the 20 canonical amino acids (Celis, 1977; Kuhn & Somerville, 1971); a further seven of the 20 can be used natively by at least some *E. coli* strains (see Table 1.2).

An invasive mirror *E. coli* or similar mirror generalist would experience strong selection to increase its growth and survival, both within hosts and in the external environment. Mirror populations in a single large animal or patch of soil could greatly exceed the numbers typically used in laboratory evolution experiments, providing substantial opportunities for adaptive evolution. Spatiotemporal gradients and other environmental heterogeneities could further accelerate evolution by selecting for different capabilities, which could promote sustained adaptation by preventing populations from becoming trapped at local fitness peaks (Baym *et al*., 2016; Kashtan *et al*., 2007). Potential targets of adaptation in mirror bacteria would include improved chemotaxis toward accessible nutrients (Adler *et al*., 1973), faster transport of nutrients across the cell envelope, improved regulation of catabolic pathways for switching between available nutrients, enhanced translocation into and infection of multicellular hosts, tolerance to acidity and reactive oxygen to survive in the phagosomes of protists and phagocytes of hosts, and tolerance to abiotic stressors such as temperature extremes and low water availability.

Evolution is typically slower to discover novel capabilities that are unrelated to existing genes. Despite their large populations, invasive mirror bacteria might take a long time to acquire some highly beneficial functions due to their initially low genetic diversity and lack of horizontal gene transfer with natural organisms. Enzymes able to catalyze novel reactions, such as a mirror nitrogenase in *E. coli*, might require millions of years to evolve, if ever, as no genes with related functions would be present in the initial mirror strain (unless introduced through human intervention). A key question concerns how long it would take for D-glucose catabolism to evolve if it was not engineered into the initial strain. A mirror *E. coli* would likely need at least four new enzymatic activities, as well as a transporter. However, the basic catalytic mechanisms would already be present in existing metabolic pathways targeting different sugar substrates. Moreover, the mirror bacterium would gain energy and fitness upon altering the substrate specificity of an existing enzyme to catalyze each of the first two steps (Shimizu *et al*., 2012). The hydrolysis of di- and oligo-saccharides such as D-sucrose and D-trehalose could be important within plants and insects, though these capabilities might be able to evolve from mutations to existing glycoside hydrolases.

An invasive mirror bacterium could diversify and fill available niches

If they spread through the environment, lineages of mirror bacteria would be subject to diverse selective pressures. Mutations that are beneficial under some conditions are often deleterious under others (Cooper & Lenski, 2000), favoring diversification into increasingly specialized strains. Even in extremely simple laboratory conditions, initially homogeneous populations of bacteria often evolve stable ecotypes that specialize on different physical, chemical, and biological aspects of their environment (Good *et al*., 2017; Rainey & Travisano, 1998). Geographic distance would also cause

mirror bacterial populations to diverge over time, even without differences in selection pressures. Increasingly fit mirror bacteria could expand at the expense of their progenitors, although this displacement would usually be slower than the initial invasion of mirror bacteria.

As previously mentioned, horizontal gene transfer between natural-chirality bacteria and mirror bacteria would be impossible. Horizontal gene transfer between mirror bacteria would be theoretically possible, but in the absence of mirror phages or conjugative plasmids it would require the uptake of and transformation by L-DNA released from mirror cells. Also, during the initial evolution of invasive mirror bacteria, the importance of horizontal gene transfer for evolution would be limited by their lack of genetic diversity. However, as mirror lineages diverged, horizontal gene transfer between them could become a more important driver of their evolution, especially if mirror phages or plasmids were present.

In summary, an invasive mirror bacterium capable of spreading in the environment would likely produce a very large population, allowing it to generate diverse mutations upon which selection would act to increase both its local fitness and its ability to colonize previously inaccessible environments. However, novel enzymes with functions unrelated to those already present would be slow or unlikely to evolve. Even if a released mirror bacterium was initially restricted to a narrow set of environmental conditions, it could evolve rapidly to expand its range. The potential for mirror bacteria to evolve and diversify underscores the biosafety and biosecurity challenges raised by their creation (see Chapter 3).

8.5 Invasive mirror bacteria could cause irreversible ecological harm

Predicting whether a mirror bacterium could colonize any given environment is challenging, and forecasting the long-term consequences of a successful invasion is even more so. Invasive mirror bacteria that become common in the environment might well eliminate some of those animal and plant species that are susceptible to infection. Less visible but equally important impacts to microbial ecosystems could lead to major and irreversible changes to ecological and geochemical processes.

We emphasize severe outcomes because they are the most consequential and cannot be excluded based on what we know from the limited available data. Given the multipronged risks to human health (Chapter 4), animals (Chapter 6), plants (Chapter 7), and natural ecosystems (this chapter), we think it is important to emphasize the breadth and depth of the problems that could be caused by mirror bacteria if they invade and become established in nature.

Invasive mirror bacteria could drive susceptible plant and animal species to extinction

Invasive pathogens are a major cause of ecological harm, and in some cases have driven species to extinction. The amphibian-killing fungus *Batrachochytrium dendrobatidis* spread from East Asia sometime in the early 20th century (O'Hanlon *et al*., 2018), leading to global declines of amphibian populations and contributing to at least 90 presumed extinctions (Scheele *et al*., 2019). In less than a decade, the invasive white-nose fungus has killed over 90% of several North American bat species (Cheng *et al*., 2021) and may drive multiple species to extinction. The oomycete *Phytophthora cinnamomi* infects ~5 000 plant species, causing a form of root rot that devastates native ecosystems

in Australia and Europe and threatens hundreds of susceptible plants with extinction (Invasive Species Council, 2023). The invasive *Ceratocystis lukuohia* fungus is rapidly infecting and killing trees of the keystone ʻōhiʻa tree (Barnes *et al*., 2018), which accounts for ~80% of the biomass in Hawaiian forests and 50% of the surface area of all woody plants on the archipelago (Fortini *et al*., 2019). Also in Hawaii, the introductions of avian malaria and bird pox were likely responsible for the extinction of a substantial proportion of the native avifauna in the late 19th century (van Riper *et al*., 1986; Warner, 1968).

An important feature of the aforementioned pathogens is their ability to infect many host species. While there are numerous examples of infectious diseases causing large reductions in host populations, diseases do not usually cause the extinction of their host species (Smith *et al*., 2006). Most pathogens require sufficient host density to survive and spread, and so pathogen frequencies generally decline once the host population falls below some threshold density (De Castro & Bolker, 2005; McCallum, 2012). For pathogens that can exploit multiple hosts or which replicate in the environment, this logic no longer applies. Persistence in the more resilient hosts or external reservoirs allows the pathogen to be repeatedly reintroduced into the more susceptible hosts.

An invasive mirror bacterium could plausibly infect a diverse set of host species (see Chapters 6 and 7), and might be able to colonize some external environments as well (this chapter). This breadth could make invasive mirror bacteria particularly ecologically devastating. Given the broad immunological deficiencies discussed in Chapters 4, 6, and 7—consequences of the complete naivety of all natural organisms to mirror bacteria—it is plausible that many susceptible populations of plants and animals would contain few or no individuals with intrinsic resistance to infection. Such populations could be rapidly driven extinct, with little opportunity for the evolution of a resistant phenotype.

Species in which preexisting resistance to mirror bacteria was fortuitously more common would likely see rapid evolution toward increased resistance to infection. While rapid evolution is observed in response to some pathogen outbreaks (Bonneaud *et al*., 2018; Di Giallonardo & Holmes, 2015; Epstein *et al*., 2016), there are important differences with the case of mirror bacteria. In particular, as previously noted, specialized pathogens typically cannot drive their host species to extinction, which allows time for the gradual evolution of traits that increase the host's resistance. For invasive mirror bacteria (or other diseases with environmental reservoirs or multiple hosts), species survival requires that some individuals are able to survive and reproduce in the face of repeated exposure from environmental reservoirs or spillover infections from other susceptible species. In vertebrates, this challenge may be further exacerbated by the likely impairment of adaptive immunity and immune memory formation (see Chapters 4 and 6); unlike most infectious diseases, an individual's fortuitous survival of a mirror bacterial infection may not provide robust protection against future infections.

Widespread infections leading to population crashes or extinction of susceptible animal and plant populations may be the most substantial cause of ecological harm from invasive mirror bacteria. We should emphasize that the degree to which different animal and plant species are susceptible to mirror bacterial infection is uncertain, and would likely depend on the specific mirror bacterium in question. Nevertheless, a mirror bacterium capable of infecting multiple species could be

ecologically devastating to an extent that is potentially much greater than existing invasive pathogens.

Disruptions to terrestrial ecosystems could eliminate key habitats and impact geochemical cycles

While the extinction of any species is unfortunate, the loss of many species simultaneously could cause severe disruptions to critically important ecosystems and the functions they provide. This is particularly true if certain "keystone" species or guilds (i.e., groups of species that perform similar functions) are susceptible to mirror bacterial infection. For example, while the invasive *C. lukuohia* fungus is unlikely to drive the ʻōhiʻa to extinction (Luiz *et al*., 2021), ʻōhiʻa are so dominant in Hawaiian forests that the loss of most mature trees would eliminate the primary habitat of most other native plant and animal species (Fortini *et al*., 2019). Coral reefs, mangrove forests, coastal salt marshes, and wetlands are likewise reliant on certain key species to stabilize the physical environment and provide habitats for other species. If these key species were susceptible to mirror bacterial infection, the result would not just be their potential loss, but also severe damage to the entire ecosystem.

While most forest ecosystems are more diverse and robust than Hawaiian ʻōhiʻa forests, a few major genera of trees dominate many global forests, particularly beech, eucalypt, pine, oak, and spruce trees. If members of these genera were susceptible to mirror bacterial infection, the results could severely damage local forest ecosystems. Forests are also important stores of carbon, containing about 450 GtC (Erb *et al*., 2018), and so the death and decomposition of even a small fraction of these trees could greatly exceed the 11 GtC/yr emitted by human activities (Friedlingstein *et al*., 2023). If only a moderate fraction of trees prove susceptible, the long-term effects on geochemical cycles might be moderated as resistant trees grow to replace susceptible ones. On the other hand, if mirror bacteria could infect most tree species and prevent their regrowth, the climate impacts could be dramatic, with atmospheric CO_2 potentially increasing on the order of 100 ppm over the course of a few years.

While not as visible as plants and animals, microbial communities within soils are also vital for many ecosystem functions. Soil microbiota are critical for the transformation and recycling of organic matter (Hoffland *et al*., 2020), nitrogen fixation (Chen *et al*., 2003), and making micronutrients available to plants (Singh *et al*., 2022). Physical, chemical, and biological disruptions to soil ecosystems are known to degrade soil quality and thereby reduce agricultural or ecosystem productivity (Eswaran *et al*., 2001), and can trigger desertification (D'Odorico *et al*., 2013). Mirror bacteria could directly impact soil communities by competing with other microbes for organic matter (to the extent it is accessible to mirror metabolism) and key inorganic nutrients like phosphorus and nitrogen, as well as by potentially harming bacterial predators and other soil fauna. Because these types of disruptions would be unprecedented, predicting the consequences is exceptionally difficult.

Global soils contain around 2500 GtC (Jackson *et al*., 2017), much greater than the 900 GtC currently present in the atmosphere and the 450 GtC in vegetation. Thus, the loss of substantial primary production combined with the potential release of stored carbon from degraded soils could

theoretically lead to a further increase in global temperatures, although the uncertainty surrounding this outcome is considerably greater than for direct impacts.

Ecological disruptions would lead to strong selective pressures on natural organisms, but some responses could be severely constrained

If mirror bacteria were to become abundant, they would exert strong selection pressure on other species. As discussed earlier, organisms susceptible to mirror bacterial infection would experience intense selection for resistance. If most individuals in a population were susceptible to infection, a population crash could ensue, limiting the population's genetic diversity and potentially harming the associated ecosystem. If extinction does not occur, then rapid evolution driven by selection among the surviving variants is a likely consequence of the population crash.

More generally, other organisms could not preemptively adapt to the presence of mirror bacteria elsewhere in the world, or even to early colonists at low abundance. Strong selection pressure would require relatively abundant mirror bacteria in the local environment. Evolution is therefore unlikely to prevent either the initial spread of mirror bacteria or mitigate any irreversible ecological harms they might cause during the first wave of colonization.

Given enough time and selection pressure, other organisms could likely evolve to prey upon or otherwise harm the mirror bacteria. Natural bacteria might have a particularly rapid evolutionary response, given their own extremely large populations and high replication rates, as well as some preexisting weapons that may be partially effective against mirror bacteria and might be refined to increase their efficacy. It also seems likely that natural bacteria would evolve to consume some mirror bacterial detritus, eventually including the mirror-chirality macromolecules that initially could not be utilized; if so, some might evolve to actively kill and consume mirror bacteria. Horizontal gene transfer might also spread such antimirror adaptations between natural strains of bacteria, albeit only once populations of mirror organisms have become abundant.

The evolution of protists and animals that prey on mirror bacteria appears more challenging. Protists and animals lack the enzymes required to degrade mirror proteins, sugars, nucleic acids, and lipids. They likely have limited or no ability to catabolize most mirror metabolites (Friedman & Levin, 2012), and some D-amino acids are toxic to many organisms (Forsum *et al*., 2008; Friedman & Levin, 2012; Yow *et al*., 2006; see also Box 1.2). These deficiencies could not be remedied through a handful of mutations, but would require the much slower evolution of novel proteins and catabolic pathways.

To give an illustrative example, *C. elegans* relies on many dozens of distinct lysozymes, glycosidases, proteases, phospholipases, nucleases, and other lytic enzymes to digest the macromolecules present in their bacterial prey (McGhee *et al*., 2007; Yilmaz & Walhout, 2016). Digesting even a simple macromolecule like bacterial-derived glycogen requires intestinal amylase and α-glucosidase to decompose the glycogen into first maltose and then glucose, followed by transport into intestinal cells via the FGT-1 transporter (Kitaoka *et al*., 2013), where glucose is catabolized into phosphorylated trioses through several additional enzymes. Evolving a similar catabolic pathway for mirror glycogen would almost certainly require a similar or greater number of

novel enzymes, and it seems questionable whether such adaptations could arise in nematodes even over millions of years.

Absent these adaptations, the consumption of mirror bacteria by many natural organisms would likely be futile or even harmful. Thus, there would be no selection pressure for increased consumption of the mirror bacteria, and indeed there might instead be selection to avoid them. It is certainly possible, perhaps even likely, that some protists and animals would eventually evolve the ability to consume mirror bacteria, but it is impossible to predict how long this would take or which taxa might acquire this ability.

It is unclear whether phages that target mirror bacteria would arise naturally. As discussed earlier, natural phages cannot infect mirror bacteria, as there is no plausible mechanism by which a natural-chirality viral genome could be replicated within a mirror bacterium. There is also no plausible natural mechanism by which a natural-chirality viral genome could be transcribed into mirror nucleic acids and translated into mirror proteins. It is therefore extremely unlikely that existing natural phages would evolve to infect mirror bacteria. However, if the mirror bacterium harbored a prophage in its genome, the prophage might be activated and evolve to become an effective predator. Also, the ultimate origins of viruses remain uncertain (Krupovic *et al*., 2019), and so perhaps mirror phages could eventually evolve *de novo* from mirror bacteria.

While natural enemies to mirror bacteria might eventually arise and help limit mirror bacterial populations, they could not plausibly drive all mirror bacteria to extinction. Given the fundamental biochemical differences between mirror bacteria and natural-chirality organisms, predation on mirror bacteria would require specialized adaptations that were advantageous only if mirror bacteria were present in the environment. Once they become established in the environment, the complete elimination of mirror bacteria therefore appears unlikely, even over evolutionary timescales, and the final "equilibrium" state that is achieved may be very different from present-day ecosystems.

Mirror cyanobacteria could profoundly alter marine ecosystems and the global carbon cycle

Photoautotrophs fix carbon directly from sunlight and carbon dioxide, and often do not require any chiral nutrients in order to grow. The production of organic matter by photoautotrophs in turn lays the foundation for almost all other life on the planet. This makes the prospect of a mirror photoautotroph particularly concerning from an ecological standpoint. For concreteness, this section focuses on the consequences of a mirror *Prochlorococcus*, though the effects of a mirror marine *Synechococcus* (a paraphyletic genus which contains *Prochlorococcus* as a subclade (Komárek *et al.,* 2020) could be similar. It appears less likely that a mirror phototroph would be created than a mirror heterotroph, given the potential biotechnological applications of a mirror heterotroph. Nonetheless, it is important to consider the possibility of a mirror prototroph, perhaps especially given interest in engineering solutions to climate change.

As discussed in Section 8.2, a mirror *Prochlorococcus* could plausibly replace native *Prochlorococcus* strains across much of the world's oceans. Natural *Prochlorococcus* growth rates under current marine conditions are typically ~0.5 day^{-1} (Kirchman, 2016), and growth is typically

limited by nitrogen (Paerl, 2018), although iron (J. K. Moore *et al*., 2001) and phosphate (Martiny *et al*., 2019) can also be limiting. Because the protists and phages responsible for almost all *Prochlorococcus* mortality (Beckett *et al*., 2024) would not efficiently target a mirror version, the mirror bacterial population would likely expand until the local concentrations of nitrogen, phosphorus, and/or iron were drawn down to levels too low to support further growth.

As nutrient concentrations decrease, so would the populations of existing phototrophs and the protists and phages that prey on them. While it is impossible to predict precisely how marine ecosystems would be affected, the consequences would likely be severe. It is extremely unlikely that natural-chirality phototrophs would be driven extinct; with the decline of many predators, some native *Prochlorococcus* strains and other phototrophs could still coexist with the mirror *Prochlorococcus* due to their greater genetic diversity and adaptation to particular niches. Nevertheless, it appears likely that a new equilibrium would be reached in which mirror *Prochlorococcus* dominated a nutrient-depleted ocean. The greatly reduced population of natural phototrophs would support a much less diverse marine food chain, as the reduction in digestible biomass would likely drive many animal species to extinction (see Figure 8.2).

The effects of mirror *Prochlorococcus* on the carbon cycle would be even more complicated. It has previously been speculated that mirror cyanobacteria would sequester vast quantities of CO_2 from the atmosphere, causing terrestrial photosynthesis to fail over a few centuries and triggering an ice age (Bohannon, 2010). This scenario does not appear particularly likely to us because the production of mirror *Prochlorococcus* biomass would be limited by the availability of other nutrients; however, it cannot be ruled out if there were substantial carbon fixation beyond that needed to produce biomass. The next few paragraphs explore these complex dynamics in more detail.

In the tropical and subtropical waters where *Prochlorococcus* thrives, the limiting nutrient is often nitrogen. As the mirror *Prochlorococcus* population expanded, it would sequester nitrogen within its own biomass. Transferring nitrogen from natural-chirality biomass to mirror biomass would not greatly change the total fixed carbon in the surface oceans, as the C:N ratio of *Prochlorococcus* of about 7:1 is broadly comparable to other living organisms (Bertilsson *et al*., 2003). Transferring nitrogen from dissolved organic matter to mirror biomass would meanwhile decrease the total fixed carbon, as the C:N of dissolved organic matter is typically closer to 15:1 (Sipler & Bronk, 2015). Transferring nitrogen from nitrate and other inorganic sources to mirror *Prochlorococcus* would also increase fixed carbon stockpiles. But because most nitrogen in the surface ocean is already sequestered in biomass and dissolved organic matter (Sipler & Bronk, 2015), it is not clear that the takeover of surface oceans by mirror *Prochlorococcus* would greatly increase the total organic matter in the surface oceans. Similar constraints would arise in regions where iron or phosphorus is limiting.

Not all carbon fixed by photoautotrophs goes into biomass. Since they are typically limited by nitrogen and managing photosynthetic fluxes is challenging, *Prochlorococcus* cells have been observed to release anywhere between 2% and 90% of the carbon that they fix back into the environment (Bertilsson *et al*., 2005; López-Sandoval *et al*., 2013; Roth‐Rosenberg *et al*., 2021). Some of this fixed carbon is released as glycolate, acetate, and other small metabolites, but much of it takes the form of complex and poorly characterized high-molecular-weight compounds (Iuculano *et al*., 2017; Kujawinski, 2011). The mirror versions of these large compounds could probably not be

degraded by existing heterotrophs. Since these compounds lack nitrogen and could not support growth in a nitrogen-limited environment, there may not be substantial selection to acquire the capability to consume these compounds. Absent consumption, this leakage could therefore result in much greater concentrations of organic carbon in the surface ocean.

Predicting how much carbon overproduction would occur in the years and decades following the release of a mirror *Prochlorococcus* is challenging. On the one hand, the standing biomass of mirror *Prochlorococcus* could be large. A recent estimate suggests that the current standing biomass of planktonic autotrophs is roughly 0.6 GtC, of which perhaps a quarter is *Prochlorococcus*; the total biomass of marine organisms is closer to 6 GtC, or about ten-fold greater (Bar-On & Milo, 2019). While these estimates are very crude, they suggest that the standing biomass of a mirror *Prochlorococcus* could potentially even exceed that of all present-day phytoplankton. It is therefore at least possible that carbon overproduction by mirror *Prochlorococcus* could be comparable to or even greater than current marine net primary production (NPP), which is about 50 GtC/yr (Field *et al*., 1998). On the other hand, given the wide uncertainty in the extent of carbon overproduction and the very slow biomass accumulation of the mirror *Prochlorococcus* population once nutrients are depleted, it is plausible that mirror *Prochlorococcus* would engage in very little carbon overproduction.

If carbon overproduction proved to be very large, then the climate consequences could be severe even over a few decades. The atmosphere contains about 870 GtC of carbon—up from the pre-industrial average of 571 GtC (IPCC, 2021)—and the removal of tens of GtC each year would lower atmospheric CO_2 to below pre-industrial levels in a matter of years or decades, and to levels below that required for terrestrial photosynthesis over hundreds of years, as speculated elsewhere (Bohannon, 2010). If carbon overproduction were instead minimal, then the initial climate effects would probably be small, though other ecological consequences would still be severe. Over longer timescales the climate would still likely be greatly altered, because biological processes play a crucial role in determining carbon storage in the deep ocean (Ridgwell & Arndt, 2015) and these processes could be profoundly disrupted by mirror *Prochlorococcus*. In particular, *Prochlorococcus* and heterotrophic bacteria are close to neutrally buoyant; the extent to which a mirror *Prochlorococcus*-dominated ecosystem would sequester carbon through the usual biological pump (Siegel *et al*., 2023) is therefore unclear. Careful modeling efforts might better discern the range of plausible climate outcomes over both decades and millenia. However, given the uncertainties in the extent of carbon overproduction and the longer-term ecological and evolutionary responses to mirror *Prochlorococcus*, substantial uncertainty will doubtless remain.

8.6 Countermeasures to invasive mirror bacteria might lessen but would not halt the ecological damage

Strategies to minimize the environmental harms caused by invasive mirror bacteria can be divided into two main categories: those that directly reduce populations of mirror bacteria, and those that reduce the adverse effects on other organisms. Neither approach appears likely to prevent widespread, irreversible environmental damage.

Biological methods could reduce but not eliminate mirror bacterial populations

Given the immense size of natural ecosystems, the only realistic methods for reducing mirror bacterial populations at a global scale are self-replicating biological agents. Human manufacture of mirror antibiotic compounds might protect or remediate small areas, but even switching the entire global production of antibiotics to antimirror compounds may not provide all humans with prophylactic treatment. As noted in Chapter 7, protecting crops through antimirror compounds appears infeasible; scaling production to the level required to protect entire ecosystems would be impossible. Other conventional means for preventing the spread of agricultural pathogens could not be performed at the required scale (Section 7.4), and could entail significant environmental damage if applied to natural environments (rather than agricultural fields).

By contrast, biological agents that prey on mirror bacteria could self-replicate and multiply in the environment with no need for manufacture at scale. Mirror phages are the simplest and most promising such biological countermeasure. A mirror phage could function as an effective predator (Weitz, 2016), substantially reducing the local abundance of its prey. Like mirror bacteria themselves, mirror phages could be spread by the movement of water, wind, and animals; spread through marine environments would be particularly rapid due to passive movement by ocean currents and turbulent mixing (see Section 8.3). The extent to which a mirror phage could spread effectively through terrestrial environments is less clear, though mirror phages could be released in many locations to assist their spread.

Despite their ability to replicate, mirror phages suffer from inherent limitations. Sustained phage replication would require the presence of a sufficiently high density of mirror bacteria in the environment. As a result, mirror phages could never prevent the initial invasion and spread of mirror bacteria into a new environment, a fact more generally true of any attempt to use specialist predators as biological controls (Lewis *et al*., 2016; Owen & Lewis, 2001). For similar reasons, mirror phages would not eliminate mirror bacteria from the environment, as the mirror phages require sufficient densities of mirror bacteria in order to replicate. At best, mirror phages would help control the equilibrium concentrations of mirror bacteria in the environment, thereby reducing, but not eliminating, infections of susceptible multicellular organisms. Such limitations are familiar from the analogous use of biological controls in the management of invasive species, where the intended aim is to maintain the density of the invasive species at a low homeostatic equilibrium (Murphy & Evans, 2009).

A further difficulty with mirror phages is that they would exert strong selection pressure on the mirror bacteria to evolve phage resistance. Inactivating the surface receptor used by a phage to enter the cell, or reducing its production, can render that cell entirely or partially resistant to that particular phage (Bohannan & Lenski, 2000; Meyer *et al*., 2012). These receptors generally have important functions for the bacteria; many are involved in acquiring nutrients from the environment. In nature, resisting predation from any given phage may be less important than retaining the function of the receptor, although resistance can sometimes evolve by altering a receptor's structure without destroying its function. Moreover, an invasive mirror bacterium could plausibly lose multiple receptors and still thrive. Though the phages themselves may evolve to overcome host resistance, and humans could synthesize mirror versions of phage known to prey upon the natural equivalent of the

mirror strain, it is likely that the coevolving mirror bacteria would diverge into multiple lineages, each preyed upon by specialized phages (L. Chao *et al*., 1977; Weitz *et al*., 2005; Williams, 2013). A single mirror phage could effectively control the population of any given mirror bacterial strain. However, as the number of strains expands, the coevolving phage lineages may become less effective at controlling the total population of mirror bacteria (Thingstad, 2000; Waterbury & Valois, 1993).

As noted in Section 5.3, using mirror phages to control mirror bacteria would present some additional risks: they would provide a mechanism for horizontal gene transfer between mirror bacterial strains and introduce novel genes and proteins into the mirror bacterial population. If an invasive mirror bacterium were already causing major ecological damage, these considerations would likely be secondary to the need to reduce the mirror bacteria and their harmful effects by whatever means available. Importantly, mirror phage countermeasures would not need to be developed in advance, as they could be swiftly generated once the target species of mirror bacterium was identified.

Other biological means of controlling mirror bacterial populations appear even more challenging while suffering from similar limitations. Protists could, in principle, be reengineered to recognize mirror bacteria, equipped with enzymes capable of degrading mirror macromolecules, and provided with metabolic pathways to utilize mirror metabolites. Such engineering is, however, beyond current abilities. Because protist species have limited ecological ranges (Grossmann *et al*., 2016), many different protist species may need to be reengineered in order to provide top-down biological control across the relevant ecosystems, which would further increase the challenge. The primary advantage of engineering protists to consume mirror bacteria is that, over the very long run, such protists might be more effective than mirror phages at controlling the total mirror bacterial population; protist predation tends to be less strain-specific and the evolution of resistance less straightforward. Engineering heterotrophic bacteria and/or fungi to produce antimirror compounds would likely be easier than reengineering protists, but also probably less effective.

In summary, if an invasive mirror bacterium were to be released into the environment, there appears to be no practical means to either prevent its spread or eliminate it from the environment. Predators including mirror phages and, more speculatively, engineered protists could restore some degree of top-down control on mirror bacteria and so help to reduce their overall numbers. However, they could neither eliminate mirror bacteria nor prevent potentially irreversible ecological changes. Indeed, the addition of human-engineered biological control agents might lead to further unexpected and irreversible ecological changes. While the production and establishment of artificial biological controls might prove to be necessary, they could neither prevent nor reverse severe ecological harms expected to result from the introduction of invasive mirror bacteria.

Germline engineering might allow the creation of plants and animals resistant to mirror bacteria

Of the many potential ecological consequences of invasive mirror bacteria, perhaps the most concerning is that a mirror heterotroph could directly infect and kill many animal and plant species to the point of extinction. Given the rapidity with which invasive microbes can spread through the

environment and the absence of biological controls or other realistic countermeasures to block the spread of mirror bacteria, protecting susceptible wild populations appears implausible.

It might be possible to genetically engineer some animals and plants to resist mirror bacterial infection (as discussed for plants in Chapter 7). Receptors might be engineered to recognize common mirror macromolecules (Cesari *et al*., 2022; June & Sadelain, 2018), synthetic mirror-responsive receptors could trigger the release of anti-mirror effectors (Weinberg *et al*., 2024; Zhu *et al*., 2022), and mirror-responsive opsonins (Banaszek *et al*., 2019) could enable existing neutrophils to find and kill infecting mirror bacteria with ROS. Any natural plants and animals that proved resilient to infection might suggest other protective mechanisms. If successfully engineered into the germline, such measures could potentially prevent mirror bacteria from establishing an infection in the protected organisms and their descendants.

While heritable immunity to mirror infection appears feasible with sufficiently advanced biotechnology, logistical hurdles would likely preclude protecting more than a tiny fraction of the millions of different plants and animals in nature (Mora *et al*., 2011). Thus, germline engineering would likely prove most effective if used to protect key crops and a few other highly valued species. Along with any naturally resistant survivors, these engineered species might be used to repopulate devastated ecosystems in order to create new functional ecosystems, akin to rewilding efforts that use functional analogs (Svenning *et al*., 2016) but on a much larger scale. Engineered resistance might eventually mitigate some of the worst effects of invasive mirror bacteria, but the resulting artificial ecosystems would be greatly impoverished, and much biodiversity would be irreversibly lost.

Contributions and Acknowledgments

Author Contributions

Authors are listed alphabetically by surname for each chapter; ordering does not indicate the level of individual contributions. The initial draft of the technical report was based on research conducted by KME, which was further developed through investigations by MLN, SBO, and DAR. All authors contributed additional research, writing, editing, or feedback for their respective chapters. DJB and JMS coordinated the compilation and review of the final report.

Acknowledgments

The authors of this report would like to thank the co-authors of the *Science* article for providing input at various stages of the technical report's creation. In addition, we are grateful to the following people who provided critical feedback on drafts of individual chapters, or, in some cases, the entire report: William J. Bradshaw, Salvadore Buse, Ivana Cvijović, John Donnelly, Seth Donoughe, Steven Erickson, Julius Fredens, Louise F.H. Funke, Jacob Glanville, Alex Kleinman, Michael McLaren, Alexander Mijalis, Sriharshita (Harshu) Musunuri, Marta Napiorkowska, Velislava Petrova, Georgia Ray, Daniel Rice, Adin Richards, Maximilian Schons, Jon Sanders, Elizabeth Shepherd, Chris Somerville, Christopher Stamper, Benjamin Stewart, Gina Suh, Joe Torres, Kyle Webster, James H.R. Wheeler, and Heather Youngs. In addition, we would like to thank Andrew Snyder-Beattie and James Wagstaff, who helped to initiate the writing of the report; and James A. Smith and Ivana Cvijović, who provided operational support in the production and review of the report. Finally, we would like to thank all of the reviewers (listed on pg iii), including those who chose to remain anonymous. Responsibility for the final content rests entirely with the authors.

Figures and illustrations presented in this report were based on concepts designed by the authors, and created in partnership with Alana McPherson and Kip Lyall at Science Journal Editors (SJE). The images are made available under a Creative Commons Attribution-Noncommercial-Sharealike License (CC BY-NC-SA 4.0).

The authors acknowledge Open Philanthropy for their support of the technical report through operational assistance, and funding to RKD, KME, GL, MLN, SBO, and JMS. DAR also acknowledges past support for work on the same topic. The authors also acknowledge operational support from the Mirror Biology Dialogues Fund, enabled by financial contributions from the Alfred P. Sloan Foundation, the David and Lucile Packard Foundation, the Gordon and Betty Moore Foundation, Open Philanthropy, and Patrick Collison. KPA and JIG acknowledge support from the National Science Foundation, Award #1901145 (RCN Build-a-Cell: An Open Community Considering & Advancing the Construction of Synthetic Cells). KME acknowledges support from NSF Award #1943141 (Towards Open and Responsive Ecological Editing). YC acknowledges support from UKRI Engineering and Physical Sciences Research Council EP/V05967X/1 (Open Plus Fellowship: Engineering and safeguarding synthetic genomes). Other authors acknowledge general support from a wide range of sources. The views expressed here are those of the individuals and not those of any organizations with which they are affiliated.

Competing Interests

VSC is a co-founder, Scientific Advisor, and equity holder in SeqCoast Genomics, LLC, and Middle Author Bioinformatics, LLC. VSC and JP are board members of the Mirror Biology Dialogues Fund. FJI is a co-founder of Pearl Bio. JDGJ is the founder and a shareholder of Norfolk Plant Sciences and Norfolk Healthy Produce. RM is a member of the advisory board for *Cell*, and the scientific advisory boards of Amgen, GlaxoSmithKline, and Janssen Pharmaceuticals. SBO is the founder and an executive at SynX Therapeutics Ltd. HS is listed as a co-inventor on patents pertaining to lactazole engineering, as well as patents pertaining to tRNA engineering to enable the incorporation of various D-amino acids. JMS is a former employee and shareholder of Teiko.bio. In the last 5 years, RKD, KME, TVI, GL, MLN, SBO, JP, DAR, JMS, and BW have, either as individuals or as project leaders, received funding from Open Philanthropy to support work that is not directly related to this report. KPA, DA, YC, TWH, JIG, REL, JWS, and NJT declare no conflicts of interest relevant to this research

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Chapter 1

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Chapter 3

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Chapter 4

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Chapter 6

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Chapter 7

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