Rethinking microbial infallibility in the metagenomics era

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Abstract
The 'principle of microbial infallibility' was a mainstay of microbial physiology and environmental microbiology in earlier decades. This principle asserts that wherever there is an energetic gain to be made from environmental resources, microorganisms will find a way to take advantage of the situation. Although previously disputed, this claim was revived with the discovery of anammox bacteria and other major contributors to biogeochemistry. Here, we discuss the historical background to microbial infallibility, and focus on its contemporary relevance to metagenomics. Our analysis distinguishes exploration-driven metagenomics from hypothesis-driven metagenomics. In particular, we show how hypothesis-driven metagenomics can use background assumptions of microbial infallibility to enable the formulation of hypotheses to be tested by enrichment cultures. Discoveries of comammox and the anaerobic oxidation of methane are major instances of such strategies, and we supplement them with outlines of additional examples. This overview highlights one way in which metagenomics is making the transition from an exploratory data-analysis programme of research to a hypothesis-testing one. We conclude with a discussion of how microbial infallibility is a heuristic with far-reaching implications for the investigation of life.

Keywords
Microbial infallibility; metagenomics; enrichment cultures; hypothesis versus exploration; bioremediation; microbial diversity

Introduction

The 'principle of microbial infallibility' is a provocative way of stating that microorganisms will always find a metabolic way to take advantage of available energy sources. It may take them some biological ingenuity and evolutionary time to do so, says the credo, but eventually the microbes will succeed. Although this basic idea has been contested since its inception in the 1950s, it has been revivified by recent discoveries of microorganisms able to exploit challenging biochemical situations (e.g. the anaerobic oxidation of ammonium or methane). More generally, this view of microbial capabilities lingers tacitly in the background of environmental microbiology and microbial biochemistry, from where it motivates many bioremediation strategies and biotechnology developments. Microbial infallibility often meshes with other maxims of microbiology, especially the 'everything is

everywhere' motto promulgated by Beijerinck and Baas-Becking. Rather than merely tolerating microbial infallibility as an implicit guide to research, we bring it to the forefront of discussion, and show how explicit reference to the principle can productively guide and integrate large bodies of research.

This review will discuss the basic insights informing the microbial infallibility principle, some findings thought to exemplify it, and the roles metagenomics can play in such investigations. Although metagenomics is often thought of as a discovery-oriented or data-driven science (e.g. Tripathi et al. 2018; Kyrpides et al. 2016), we distinguish exploration-driven metagenomics from hypothesis-driven metagenomics. We focus on bioenergetic predictions that have been realized with enrichment cultures while being implicitly and explicitly guided by assumptions of microbial infallibility. Further exploratory metagenomics has both refined and generalized such findings. We suggest that microbial infallibility as a broad heuristic still has contributions to make to microbial ecology and evolution in this era of big science.

Historical background

Microbial infallibility was first proposed by Ernest Gale (1914–2005; Figure 1) in his 1947 book, *The Chemical Activities of Bacteria*, where he argued that:

> 'Somewhere or other some organism exists which can, under suitable conditions, oxidise any substance which is theoretically capable of being oxidised' (1951, p. 5).

Figure 1: Ernest Gale and *The Chemical Activities of Bacteria* (3rd ed, 1951). First published in 1947, then again in 1948.
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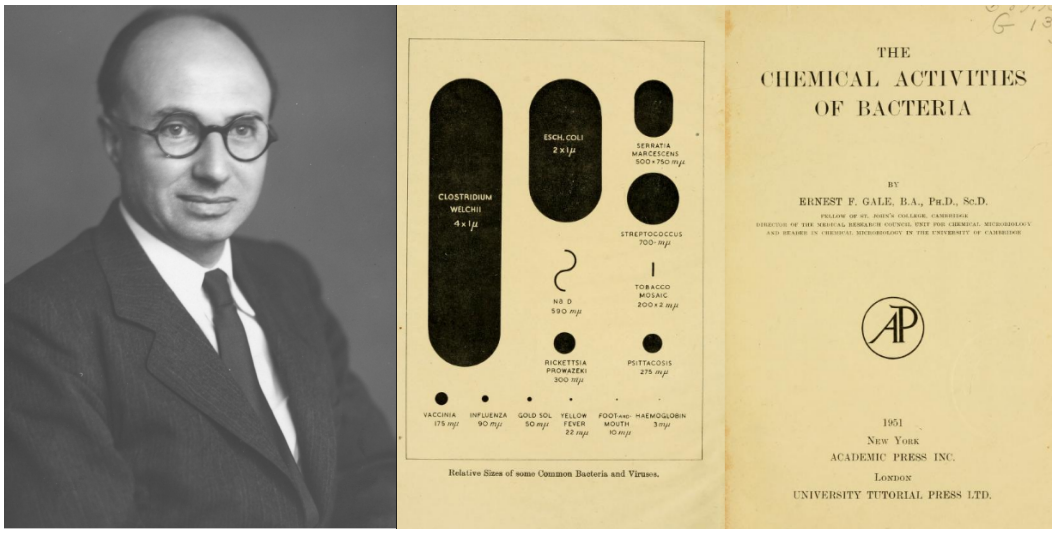

Gale ran the Cambridge (UK) Biochemistry Department after Marjory Stephenson's famed occupancy of that role. In fact, he was her protégé and PhD student. He in turn supervised mitochondrion iconoclast Peter Mitchell's PhD thesis, especially after Mitchell's first submission was a rather crushing 'revise and resubmit' (Prebble and Weber 2003). Gale himself was not much of a theorist, but an experimentalist. Nonetheless, his experiments were extensive (amino acid deamination and

decarboxylation, and antibiotic modes of action) and they led him to offer generalizations from experience. One such generalization was the 'microbial infallibility' conjecture. He did not use these words, however. Who did?

That honour belongs to Martin Alexander (1930-2017; Figure 2), who – even as he attempted to criticize the general idea of microbial infallibility – ended up immortalizing it in a very catchy formulation:

> 'It is usually regarded as axiomatic in microbial ecology that … some organism in nature … will utilize as carbon or energy source any organic compound. This concept, commonly taken as ecological dogma, may be termed the "Principle of Microbial Infallibility"' (1964, p. 246; see also Alexander 1981, p. 136)

Figure 2: Martin Alexander.
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Alexander worked at Cornell, doing 'biochemical ecology' of soil. His textbooks were hugely popular. In the 1964 paper in which he discusses microbial infallibility, Alexander also criticizes the 'enumeratomania' of microbial ecologists, or their tendency to count organisms rather than formulate principles or make generalizations about organismal occurrence or behaviour. Yet, despite his fondness for principles, he did not give much credence to microbial infallibility, because of 'the surprising lack of catabolic omnipotence of microbial communities' (1981, p. 136). In other words, he was making the rather prosaic observation that microbes are fallible. He considered this to be surprising simply because it was the opposite of standard expectations (which he saw as unfounded belief).

Alexander thought that his newly coined version of the fallibility principle was applicable to various natural products, because some of these took considerable time to degrade (e.g. humus, hydrocarbons). He put hydrocarbons and peat on this list because of their build-up underground or underwater, which he thought meant microorganisms could only consume these substances in the presence of oxygen. It is now known that diverse bacteria do catabolize a wide range of hydrocarbons in anoxic environments (e.g. Heider et al. 1998). Nevertheless, the copious accumulation of complex organic compounds

(e.g. hopanoids) in natural environments serves as an indicator of at least a degree of difficulty in organisms accessing them for consumption.

However, it was human-made compounds on which Alexander focused. He thought that 'microbial fallibility has been revealed most dramatically' by the persistence of pesticides and herbicides in the soil (Alexander 1964, pp. 246-7). He saw this as not just a matter of fallible microbes but of truly recalcitrant molecules. While he also mentioned detergents and plastics as other examples of human-made recalcitrant molecules, it was his discussion of pesticides and herbicides that gained the most attention and that he raised as the most serious for their potential effects on humans (Alexander 1981). He was not too worried about the environmental impact of plastics because he thought they were merely 'aesthetically undesirable' (1981, p. 136), which was a common view at the time.

Alexander's own investigations found 'extensive' evidence of degradation of DDT by microbes (Pfaender and Alexander 1972; Focht and Alexander 1970), but he believed they were just breaking it down and not gaining energy or carbon from it unless co-substrates were added. This degradation without growth was known as 'co-metabolism' (Horvath and Alexander 1970). Alexander argued that there would be selective disadvantages to carrying out the difficult and slow degradation of recalcitrant compounds with little metabolic gain from it, which would inevitably lead to the extinction of such organisms (Alexander 1981; Pfaender and Alexander 1972).

In a critical reflection on these arguments, Raymond S. Horvath (1943-1984), a sometime collaborator with Alexander, expressed doubts about the microbial fallibility conclusions. He suggested interpreting them as limitations of the experimental setups rather than as indications of microbial limitations. In Horvath's words, 'The evidence for molecular recalcitrance appears to stem from human fallibility rather than microbial fallibility' (Horvath 1972, p. 152). He suggested that copious data showing microbial abilities for degrading pesticides despite no gain of energy or carbon (in other words, incomplete metabolism or co-metabolism) were sufficient to show that 'the concept of molecular recalcitrance may no longer be valid' (Horvath 1972, p. 153). It might be thought that findings in more recent years of microbial growth on pesticides both with and without the addition of co-substrates (e.g. Holliger et al. 1999; Wang et al. 2010; Ortíz et al. 2013) vindicate Horvath's misgivings about both 'fallibility' and 'recalcitrance'. Likewise, growing – albeit contested – evidence for plastic consumption in microorganisms (e.g., Yoshida 2016; Yang et al. 2015; Gambarini et al. 2021) might knock back another of Alexander's examples of recalcitrance.

Despite counterarguments and counterexamples, bioremediation microbiology continues to be a stronghold for infallibility assumptions. These assumptions are apparent in the underlying expectation that cleanup of, for example, oil spills or dioxins can be achieved by microbes, even if this requires manipulation of the contaminated environment by the addition of other compounds to get full results (e.g. Ahn et al. 2008; Tyagi et al. 2011). Biostimulation does not contradict the general notion of infallibility, but simply specifies or even optimizes the conditions in which the relevant metabolism can occur. In addition, doubts about the very existence of co-metabolism have been raised (e.g. Hulbert and Krawiec 1977; Wackett 1996), especially because some of the primary evidence for degradation but not growth came from an inability to isolate and grow organisms in pure culture, as Alexander himself admitted (e.g. Alexander 1981). As is well-known now, the

problems of pure culture do not mean the relevantly metabolizing organisms are non-existent; in addition, increasing findings of metabolic interdependence between microorganisms have further challenged Alexander's evolutionary reasoning.

These points were broadly articulated by famous microbial biochemist, Patricia Clarke (1919-2010) (Figure 3), who chimed in on discussions of 'this apparently theological doctrine' of infallibility to say that:

> 'Alexander … was perhaps excessively gloomy about the prospects for microbial evolution. Part of the reason for this was that most of the studies on microbial metabolism and adaptation had been carried out with pure cultures. It is now known that several microbial species may be involved in the complete biodegradation of a single organic compound. Adaptation to novel growth substrates may also involve more than one bacterial species' (1980, p. 387).

Figure 3: Patricia Clarke.
Image used with the permission of The Royal Society (Great Britain), from *Biographical Memoirs of Fellows of the Royal Society*, Patricia Hannah Clarke, by W. J. Brammar, Vol. 61 pp. 39-51, 2015; permission conveyed through Copyright Clearance Center, Inc.

Even in Alexander's era, it was known that microorganisms can work together to overcome the energy limitations of particular substrates. Syntrophic interactions, which are cooperative interactions between species that provide a trophic benefit to both partners, are widespread in the microbial world (McInerney et al. 2009; Schink 1997). For example, classically defined syntrophic interactions occur between fermentative bacteria and methanogenic archaea, which are also found living independently. When syntrophic, their metabolic interactions are based on the transfer of electrons between the microbes, usually through the exchange of H_2 (or formate). The maintenance of extremely low H_2 concentrations by methanogenesis is what allows the syntrophic bacteria to conserve energy through the oxidation of energetically unfavourable compounds, including alcohols (e.g. ethanol) and short chain fatty acids (e.g. propionate) (Stams and Plugge 2009; Schink 1997).

Clarke is suggesting that ongoing discussions of infallibility should take into account such partnerships – already known in the 1950s and 60s, and now known to feature in the degradation of some of Alexander's 'recalcitrant' substrates (see Morris et al. 2013). But what does Clarke mean when she says 'apparently theological'? She is quite probably referring to the papal infallibility doctrine promulgated by Pope Pius IX in 1870. It announced the impossibility of error in popes, and positively asserted the perfection of papal edicts about, for instance, the assumption of Mary and immaculate conception. Alexander obviously used the term scornfully: infallibility was as non-credible in microbes as in popes. He repeatedly calls the microbial version mere 'dogma' (Alexander 1964).

Clarke may have felt some need to defend Gale against Alexander's criticisms. She also trained in the Cambridge Department of Biochemistry, a few years behind Gale, but did her main work at UCL, where she demonstrated repeatedly the evolutionary adaptiveness of microbes in new chemical environments (e.g. Clarke 1980). But more importantly, she was part of a remarkable tradition of experimental metabolic evolution, in which bacteria were understood as 'biochemical experimenters' (Stephenson 1949, p. xi). Human experimenters, such as Clarke, Robert Mortlock (1982), and Edmund Lin (Lin et al. 1976), carried out environmental manipulations to see if organisms such as *Klebsiella* and *Pseudomonas* could evolve new metabolic capacities. These studies produced a great deal of insight into the molecular mechanisms of evolution – the pathways enzymes had to take to be able to use 'unnatural' energy sources. Researchers first looked at loss of function, then gain of function, with a focus on changes in gene regulation that led to loss of repression of enzyme activity (see O'Malley 2018). However, none of these researchers explicitly addressed microbial infallibility, despite Clarke's invocation of the concept.

The realization of an infallibility prediction: anammox

Most of the work from the 1960s to the 1980s on the experimental evolution of metabolism focused on organic compounds; indeed, Alexander had invoked fallibility only that far, without paying broader attention to how organisms might – or might not – use inorganic compounds as reductants in energy metabolism. In the 1970s, using standard energetic calculations, a more encompassing challenge was articulated. It came from Engelbert Broda (1910-1983; Figure 4), an Austrian chemist who moonlighted as a KGB spy (see, e.g. Brinson and Dove 2014). He was interested theoretically in the evolution of metabolism, and argued that if it is the case that microbes are very versatile, and that they evolve, then it should be expected that they can metabolize *anything* that provides an energetic payoff. Although he did not use the language of infallibility, his famous paper, 'Two kinds of lithotrophs missing in Nature' (Broda 1977), is often taken to invoke it implicitly as he predicted organisms that should exist because of energetic benefits.

Figure 4: Engelbert Broda. The picture behind him is of the Viennese physicist, Ludwig Boltzmann, renowned for his work on statistical mechanics.
Image reproduced with kind permission of the Österreichische Zentralbibliothek für Physik/ Austrian Central Library for Physics.

Broda predicted the undiscovered and unsought existence of (1) photosynthetic anaerobic ammonia bacteria, and (2) bacteria oxidizing ammonium anaerobically with nitrite (or nitrate). He made these predictions on the basis of free energy calculations, although it was also known empirically that large amounts of ammonium are missing in oceans (meaning that something should be consuming it). Thermodynamic conjectures about potential metabolic pathways were common well before Broda, and had guided experimental work such as Stephenson's from the 1920s to the 40s (Oren 2015). Although Broda's predictions did not immediately initiate any searches for the missing organisms, his paper was rediscovered in the early stages of a research effort that turned one of the organisms and its predicted bioenergetics into a major success story.

It is Broda's second missing organism that has become the vindicating one. It involves the celebrated discovery of anammox bacteria (Kuenen 2008), which combine nitrite reduction with ammonium oxidation. The existence of such organisms was also suggested by anoxic ocean niches exhibiting ammonium and nitrate loss, but the gain of nitrogen gas (Hamm and Thompson 1941; Richards 1965). When an analogous situation was detected in wastewater, the hunt was on to find the culprits (Mulder et al. 1995). This was ultimately achieved with cultures of samples from wastewater treatment plants, grown with supplements of ammonium and nitrite (van de Graaf et al. 1996; Strous et al. 1998; Jetten et al. 1999).

Found only within the Planctomycetes, anammox bacteria turn out to be rather strange organisms with a membrane-bound metabolic organelle that has unusual membranes, made of ladderanes. As well as enabling metabolism, the organelle contains hydrazine, once used by humans as an explosive rocket fuel. Hydrazine is one of the intermediates formed by anammox bacteria, along with nitric oxide. The end product of this pathway is dinitrogen gas (Kartal et al. 2013).

Anammox continue to require enrichment bioreactors to study them. The success of this cultivation-based approach has made it possible to search for genomic traces of such organisms in natural environments with metagenomic tools (see below). Anammox are now known to be globally important in the nitrogen cycle, and entire wastewater treatments have been commercialized around them. But it was fundamentally the enrichment method, based on biochemical predictions and environmental calculations, that led to such a remarkable discovery. We will return to the significance of this method below.

What about Broda's first prediction? This was of an organism that uses ammonium as an electron donor in anoxygenic phototrophy and fixation of CO_2. No trace of that exact organism has been found so far, but phototrophic nitrite oxidizers have been identified, also via enrichment culture (Griffin et al. 2007; Schott et al. 2010; see Oren 2015 for discussion). Despite a few adjustments to Broda's predictions, nobody can deny how beautifully the second prediction of anammox was realized. We might take that as at least an indication we should think seriously about microbial infallibility and its corollaries.

'Everything is everywhere' and infallibility expectations

Whether about anammox or other capabilities, infallibility considerations also draw on the Baas-Becking and Beijerinck maxim of '*everything is everywhere*: but *the environment selects*' (de Wit and Bouvier 2006; O'Malley 2008). A strong interpretation of 'the environment selects' is in fact a restatement of microbial infallibility: the environment offers certain resources, and organisms with the ability to use those resources will evolve in or disperse to such environments, modifying those environments as they occupy them. If the biogeography of microorganisms were to be severely restricted by dispersal limitations, then it would be unwise to expect them to be available for any localized resource (in the same way we do not expect koalas to turn up on ecological timescales in eucalyptus plantations in California without human intervention). Although many geographical restrictions to microbial distributions of taxa have been identified (e.g. Papke et al. 2003; Whitaker et al. 2003; Power et al. 2018), it does not seem that these constraints cut across metabolic capacities and other gene-based traits (Louca et al. 2016; Fondi et al. 2016). The far-flung distribution of metabolic pathways such as anammox backs up microbial infallibility claims, and continues to support at least a metabolic view of everything is everywhere.

Beijerinck, the early source of this basic notion of the metabolic aspects of microbial biogeography being largely environmentally determined, might be thought of as an early source of infallibility claims. These views even recently have been an important motivator of the quest to find the microorganisms and pathways carrying out manganese oxidation. Following his own predictions and after 'much trouble', Beijerinck believed he had isolated and cultured the relevant organisms (Beijerinck 1913). He suggested they were chemolithoautotrophs, but it took more than a century to show definitively that Beijerinck was right. Numerous studies over subsequent decades provided supporting but not conclusive evidence (Bromfield 1956; van Veen 1973; Emerson et al. 1982; Thamdrup et al. 1994; Tebo et al. 2005). Recently, using enrichment culture and successive refinements of the subsequent community, Yu and Leadbetter managed to sequence the genomes of a two-species consortium, at least one member of which gained energy from oxidizing manganese while fixing carbon dioxide (Yu and Leadbetter

2020). Only one of these organisms could be isolated and cultured, but several lines of evidence supported inferences about the full pathway and its mechanisms. Although the particular culmination of this quest was spurred on by chance observations in the laboratory (of manganese oxides coating a piece of glassware), the primary driver of the inquiry was Beijerinck's 'infallibility' claim based on his energetic calculations. We will show how this conceptual framing has particularly high payoff in the current era of large-scale investigations in microbial ecology.

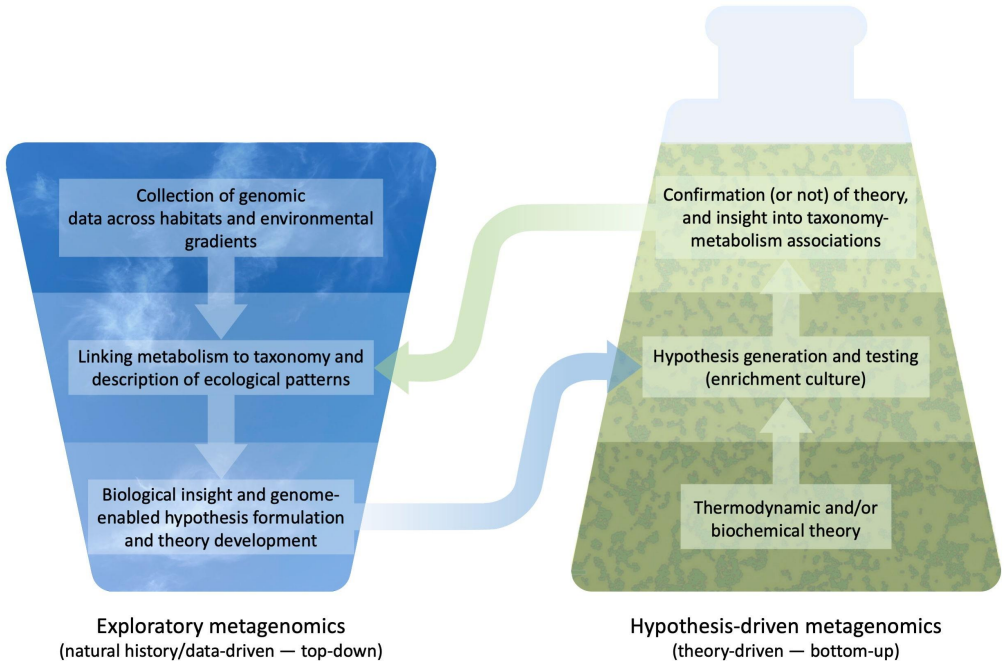
The metagenomics era

It is now well-known how microbial ecology and environmental microbiology have taken advantage of sequencing and analysis of environmental DNA from natural microbial communities (Handelsman et al. 1998; DeLong 2009; Grossart et al. 2020). But what role does the concept of microbial infallibility play in such efforts? We suggest that some of the most emphatic success stories of metagenomics have been guided by microbial infallibility expectations. These are not 'blind' discovery stories, in which random environmental sampling and DNA sequencing revealed unexpected metabolisms and community structures. Our discussion will focus on the explicit and implicit projections of microbial infallibility that guided how these investigations proceeded and the methods that were used.

It is useful here to discuss metagenomics as having two interacting modes (see Figure 5):

1. Exploration-driven: scrutinizing metagenomes for organism and metabolic pathway diversity and distribution;
2. Hypothesis-driven: using metagenomics to test and further elaborate biochemical and thermodynamic predictions.

Figure 5: Exploratory and hypothesis-directed metagenomics: their characteristics and interplay (credit: Michel Durinx, centimedia.org)



Exploration-driven metagenomics usually deals with samples of communities from environments of interest, and patterns are explained after they are found. Particular populations might then be the focus once there is an explanatory target (i.e. the organisms carrying the genes of interest). Hypotheses can be generated from these explanations, but confirming them experimentally is usually left for subsequent research. Early ocean metagenome studies exemplify this approach. The analysis of the Sargasso Sea metagenome revealed vast amounts of genetic and organismal diversity and novelty in what had previously been regarded as a low-diversity environment (Venter et al. 2004). Genomic scrutiny of microbial communities living at different depths of the North Pacific water column showed the promise of combining metagenomic and biogeochemical perspectives (DeLong et al. 2006). As well as discovery of novel or unexpected phenomena, exploratory metagenomics can expand knowledge about already-known biogeochemical processes and environments. For example, even though a rich body of geochemical, biochemical and genomic information already existed about dimethylsulfoniopropionate (DMSP) transformation in the ocean, a more directed form of exploratory metagenomics is currently elaborating on that previous body of work. New genes, new taxa and their relative abundances have suggested novel biogeochemical explanations of DMSP that now need investigation (Moran et al. 2012), and which could have considerable relevance for climate change (Kiene et al. 2000).

Examples of recent and focused exploratory findings with clear relevance for ‘infallibility’ claims include marine microorganisms with inferred capacities for metabolizing the increasing amounts of ‘recalcitrant’ organic matter flowing into the sea (Colatriano et al. 2018), and the evolution of plastic degradation metabolism in global ocean microbiomes (Alam et al. 2020). Elsewhere, metagenomic examination of environments producing manganese oxides has generated insight into not just the taxa involved but also the spatial structure of the niches and biogenic sequestering of water-contaminating metals (Sjöberg et al. 2020).

Some exploratory metagenomic investigations may then go on to use culturing to decomplexify the focal community and reveal more clearly its genomic scope. For example, in one study, the explicit ‘doctrine of infallibility’ expectation – that soil microbes would yield novel lignocellulose-degrading enzymes – was stymied by the sheer complexity of the original sample’s metagenome (DeAngelis et al. 2010). The authors turned to enrichment culturing to show that organisms with genes for such enzymes could be identified from a massively simplified community – a strategy that has great promise for applications in fuel biotechnology. Studies such as these are exploratory, even though they constrain the scope of that exploration by focusing on certain genes and organismal capacities in the context of infallibility. Such research may go on to suggest hypotheses that frame subsequent experimental work and lead to ongoing exploration.

In some cases of initially exploratory work, hypotheses end up being tested via laboratory experiments and environmental assays. A breakthrough instance of this strategy involved the discovery of bacterial rhodopsin genes in ocean metagenomes (Béjà et al. 2000). This primary finding could then be characterized experimentally, with the expression of these genes in a laboratory model system, *Escherichia coli*, since the native organisms were not cultured at the time (and still remain uncultured). Additional experimentation with environmental samples containing the relevant bacteria confirmed the photochemical potential of the membrane protein (Béjà et al. 2001). A great slew of

further metagenomic exploration then revealed the diversity and abundance of rhodopsin-bearing microorganisms in sunlit waters (e.g. de la Torre et al. 2003; Venter et al. 2004; Sharma et al. 2008). Ultimately, however, pure-culture experimentation was required to confirm that the proteorhodopsin genes made an energetic difference to their organismal bearers, and in exactly which conditions that occurred (Giovannoni et al. 2005; Gómez-Consarnau et al. 2010; Steindler et al. 2011).

The hypothesis-driven mode of metagenomics on which we focus (Figure 5) begins from the other direction. It starts with theoretical claims, which are primarily about energy gains from particular biochemical pathways, and it often uses enrichment culture metagenomics to test particular hypotheses. This approach to metagenomics might also generate new hypotheses and suggest further exploratory work that will support those additional claims. That subsequent exploratory work can enable the generalization of initial findings.

We see these two modes – exploratory and hypothesis-driven – as complementary, and not necessarily occurring in a specified order. They may repeatedly interact in longer term investigations. Microbial infallibility is most obviously implicated in the hypothesis-driven mode, in which theoretical calculations about organismal use of environmental chemicals and energy potentials are tested by carefully structured experimental observations in the context of metagenomic inquiry. We show how this works in two cases: comammox, and the anaerobic oxidation of methane. But these findings have been followed by additional exploratory work that has expanded and revised the original theoretical claims, which has then led to new hypotheses and explanations. In addition, metagenomic updates of the anammox story have gone beyond earlier enrichment culturing to produce broader generalizations.

The comammox story

Since Sergei Winogradsky's discoveries in the late-nineteenth century, nitrification has been understood to involve a division of labour between one group of organisms oxidizing ammonia to nitrite and a second group oxidizing the nitrite to nitrate. Even Winogradsky found this separation perplexing, given the potential advantages for one organism to carry out the whole process (1891, in Oren 2021). Costa et al. (2006), inspired further by Broda, postulated on the basis of kinetic optimality that an organism with both nitrification pathways (ammonia to nitrite, then nitrite to nitrate) would eventually be found because

> 'shortening long pathways could … increase growth rate. However, this would reduce growth yield if the shorter pathway has fewer ATP-generating steps' (Costa et al. 2006, p. 213).

They called this hypothetical organism 'comammox'. The argument implies that because of the energy advantage, organisms 'should' exist, which is basically a claim about microbial infallibility. A more precise secondary hypothesis would be that genes for both activities should be detected in a single organism.

Comammox organisms with such a genome were indeed discovered in 2015 (Daims et al. 2015; van Kessel et al. 2015). They are *Nitrospira* species, in which ammonia and nitrite oxidation are no longer split between organismal lineages. These organisms have advantages in oligotrophic or low-flux environments, whereas the 'canonical' two-lineage syntrophy outcompetes them in more ammonia-rich environments (Kits et al. 2017). This means slow-growing but high-yield populations of nitrifiers are more likely to be

comammox (Costa et al. 2006). Although their discovery does not change the broader details of how the biochemistry of the nitrogen cycle is understood, it does illustrate effectively how bringing theoretical predictions, metagenomics and enrichment culture together produces not only novel findings but also theoretical confirmation.

How were comammox bacteria discovered? This research success story provides an illustration of what we mean by hypothesis-driven metagenomics. It combines microbial infallibility predictions (about kinetics and thermodynamics), enrichment culturing, and exploratory metagenomic analysis. Initial metagenomics can help indicate which samples are worth growing as enrichment cultures that can test the hypothesis; targeted metagenomic analyses then confirm the enrichment cultures have selected for comammox; and finally, further exploratory but focused metagenomics can reveal the diversity, distribution and abundance of such organisms.

These steps play out in different combinations of exploration- and hypothesis-driven strategies. In one of the discoveries, Daims et al. (2015), driven by comammox predictions, analysed samples from abandoned oil wells in Chechnya, and grew enrichment cultures based on 'the intriguing possibility that the *Nitrospira* population might be responsible for both ammonia and nitrite oxidation' (Daims et al. 2015, pp. 504-505). They then sequenced the metagenome of the enrichment to identify the organism involved. The same study also carried out additional metagenome analyses to detect comammox markers in wastewater and ground metagenomes. *Nitrospira inopinata* is the only isolated comammox so far, and its 'fundamental niche' is soils, sediments and biofilms that have low levels of ammonia and are oligotrophic (Kits et al. 2017; Sakoula et al. 2021).

In a simultaneous study making another discovery of comammox's existence, van Kessel et al. (2015) used metagenomics of enrichment cultures to show how environmental samples from earlier exploratory work had previously been analysed to give the wrong results. 16S or relevant ammonia (and methane) oxidation genes had been mistakenly binned with nitrite-oxidizing or methane-oxidizing bacteria, and comammox thus escaped detection. The study identified two species of fully nitrifying *Nitrospira*, which were keeping close company in the enrichment with anammox bacteria as both groups competed for nitrite.

Subsequent studies illustrate what we mean by the enhancement of hypothesis-driven work by additional exploration. Metagenomic analyses of environments in which comammox are detected have revealed the rapid niche divergence of these organisms, the role of mobile genetic elements in such divergence, and the particular innovations underlying the global ecological success of comammox *Nitrospira* over their classically syntrophic relatives (e.g. Camejo et al. 2017; Palomo et al. 2019; 2018).

In a pithy recapitulation of the now copious findings of such efforts, Kuypers says,

> 'the comammox discovery is proof that if a process is energetically feasible, it will be performed by a microorganism or a microbial labour union somewhere' (Kuypers 2015, p. 488).

This sounds like microbial infallibility to us. The 'labour union' aspect reprises Clarke's earlier defence of microbial infallibility against Alexander (even though comammox turns out to be a labour union-busting organism).

The expanding tale of methane oxidation

Because methane is not a very reactive hydrocarbon in the absence of oxygen, it was long believed that methane oxidation could not be carried out anaerobically. However, a profusion of recent findings has shown how wrong this view was, and how ingenious microorganisms can be in finding ways to overcome energetic challenges. The initial impetus for contemporary discoveries came from geochemical knowledge of the sulfate-methane transition zone on ocean floors (Barnes and Goldberg 1976; Reeburgh 1976). In order to explain the disappearance of methane from the water column, Zehnder and Brock (1979) predicted that methane consumption occurred, and that it would be carried out by a consortium. They argued that methane oxidation would not be a 'simple back reaction' from methane production in a single organism, although they did expect that methane formation and oxidation would occur simultaneously. The experiments they conducted suggested very strongly that methanogens are involved with sulfate reducers and that one aspect of this interaction is anaerobic methane consumption.

Zehnder and Brock's predictions, plus a supporting body of subsequent field and laboratory findings (e.g. Hoehler et al. 1994), motivated a metagenomic quest for the relevant organisms and activities in anoxic sediment samples. In 2004, Hallam et al. searched for and detected genes in marine sediment samples that could be attributed to the anaerobic oxidation of methane by archaea working in syntrophy with sulfate-reducing bacteria (Hallam et al. 2004). This analysis supported the 'reverse methanogenesis' hypothesis, which involves the reversal of the pathway for producing methane. However, its occurrence in a syntrophy accords with Zehnder and Brock's (1979) argument against a 'simple back reaction'. Subsequent metagenomic analyses have filled in missing genes and – again with the help of enrichment cultures – shown how the standard methanogenesis pathway may have been modified (Wang et al. 2014).

Further combinations of metagenomics and enrichment cultures have allowed a plenitude of additional predictions and experimental demonstrations of more diverse and profitable ways to realize the anaerobic oxidation of methane than the sulfate-driven syntrophic version. Anaerobic methane oxidation coupled with denitrification should produce more energy. Expectations of finding the relevant organisms 'missing in Nature', given the energetic advantages, were explicitly about infallibility (Raghoebarsing et al. 2006, p. 918; Welte et al. 2016). Using a culture enriched with nitrate and nitrite as electron acceptors, and methane as the donor, two apparently dependent organisms were discovered to be oxidizing methane anaerobically in concert: an archaeon and a bacterium (Raghoebarsing et al. 2006). However, subsequent enrichments revealed much more precisely that both organisms could independently combine distinct pathways for anaerobic methane oxidation with nitrite/nitrate reduction. Only one of these organisms (the archaeon) was using reverse methanogenesis and was not denitrifying (Haroon et al. 2013), whereas the bacterium, *Methylomirabilis oxyfera*, was pursuing an initially obscure nitrite-driven process (Ettwig et al. 2008; 2009).

In a remarkable twist to the story, Ettwig et al. (2010) analysed the metagenomes of the enrichment cultures to find that the bacteria (*M. oxyfera*) were oxidizing methane *with* oxygen under anoxic conditions. The enrichment metagenome showed that some expected denitrification genes were missing, which eventually led to the conclusion that the bacterium was actually carrying out intracellularly aerobic oxidation of methane via the production of oxygen as an intermediate (Ettwig et al. 2010). Easy to overlook and of

considerable evolutionary importance, this finding is an elegant illustration of the interplay between infallibility expectations and metagenomics. Perhaps in this instance, an infallibility interpretation is retrospective rather than predictive, but it still helps makes sense of an otherwise puzzling situation. Producing oxygen in the way these organisms do is thermodynamically feasible, even if not a routine metabolic occurrence, and so detecting these organisms might be construed as another vindication of the basic principle.

Enrichment experimentation has also elucidated competitive relationships between the various organisms found in similar geochemical niches. To gain insight into nitrate-dependent anaerobic methane oxidation, Haroon et al. (2013) had to add ammonium to their bioreactor so that anammox (*Kuenenia*) would feed off that and enable the nitrate-reducing anaerobic methane oxidizer of interest (the archaeon *Methanoperedens nitroreducens*) to out-compete the nitrite-reducing anaerobic methane oxidizer (*M. oxyfera*). Without the ammonium and anammox dynamics, the latter would otherwise take over the enrichment culture. These environmental manipulations of the enrichment thus add an explanatory dimension to the observations that metagenomics can produce, by revealing very specific niches and particular competitive advantages.

As enrichment cultures and metagenomics continued, further details emerged of the pathways implemented in anaerobic oxidation of methane. The archaea coupling reverse methanogenesis to sulfate reduction can do so without any assistance from the sulfate-reducing bacteria, although there are still advantages to intimate cohabitation of the same environment (Milucka et al. 2012). The detection of extracellular electron transfer between these species helps explains the relationship (e.g. Scheller et al. 2016). Alternative electron acceptors may be involved, including iron and manganese (e.g. Ettwig et al. 2016; Cai et al. 2018; Leu et al. 2020). These prolific ongoing discoveries illustrate a key point of our discussion, which is that all these subsequent discoveries were made with metagenomic analyses of enrichment cultures, structured by hypotheses about what is possible metabolically and – if sufficient background knowledge exists – what genes might be involved. The highly profitable combination of these methodological steps bring us back to metagenomic updates of the original anammox revelations.

Anammox redux

The anammox discovery was made on the basis of enrichment cultures, but once the first organisms were detected and their genomes sequenced (e.g. Strous et al. 2006), these sequences could be looked for metagenomically. Doing so was impossible prior to the revelations of the original enrichments. Knowledge of the anammox world has been vastly expanded by metagenomics of environmental samples, which show that there is an even greater abundance and diversity of these organisms than expected, and that they play major biogeochemical roles (Oshiki et al. 2016). Anammox are found not just in wastewater, but also freshwater and seawater, as well as sediments and soils (Strous et al. 2006; Gori et al. 2011; van de Vossenberg et al. 2013; Wu et al. 2019), and their genomes may vary considerably depending on their niches. More extensive analyses of nitrate-rich sites reveal anammox organisms have many secondary metabolites that seem to be antimicrobial and may explain why there are so many of these organisms despite growing more slowly than potential competitors (Ludington et al. 2017).

Enrichment cultures, combined with metagenomics and metatranscriptomics, continue to deliver new findings about the versatility of anammox. One recent candidate possesses an ability to reduce nitric oxide rather than nitrite, and this finding then generates hypotheses about what the ancestral anammox pathways may have been (Hu et al. 2019). Other hypothesized anammox organisms continue to exist only in the form of abstract thermodynamic possibilities (see Kartal et al. 2013). For example, sulfate-dependent anammox (in ‘t Zandt et al. 2018) has still to show itself, despite some environmental indications and potential thermodynamic possibilities involving syntrophy. But no matter whether these and other predicted organisms are found, enrichment cultures will be utterly key to ongoing searches, and enrichment metagenomics a major source of additional information about anammox physiology and diversity.

Hypothesis-driven metagenomics with enrichment cultures

But isn’t metagenomics the genomic analysis of ‘naturally occurring populations and communities’ (e.g. Tringe and Rubin 2005, p. 805)? This definition of metagenomics, which is intrinsically exploratory, does not quite fit what has occurred in the success stories above. Why use enriched environments, which are artificial and human created, instead of sampling directly from nature?

To answer such questions, we need to look more closely at the nature and purpose of such cultures. Enrichments are carefully designed selective environments that match and thus test theoretical expectations. These cultures can reveal not only the relevant metabolic pathways and the organisms that actualize them, but also clarify relationships between organisms (e.g. Haroon et al. 2013). Enrichments may also help explain the structure and dynamics of particular communities, and lead to greater explanatory insight into the distribution and abundance of such organisms beyond the laboratory (e.g. Palomo et al. 2018). Even for the purposes of preliminary exploration, enrichment cultures can allow the detection of relevant genes for novel enzymes, as in the lignocellulose search discussed above (DeAngelis et al. 2010). Many of the discoveries we note above simply could not have been made by exploratory metagenomics alone. For all these reasons, enrichment cultures combined with metagenomic analyses before, during and after the culturing process will lead to more depth in understanding the relevant communities and their niches.

Now obviously, metagenomics is not always or even often concerned with carefully designed enrichment cultures, but much of the crucial work for the major discoveries discussed above did involve both methods. This combination of approaches may ultimately enable the holy grail of isolation and pure culture on which classic microbiological experimentation depends (e.g. the comammox isolation of Kits et al. (2017) or the chemoautolithotrophic manganese oxidizer in Yu and Leadbetter (2020)). However, exploratory metagenomics can both precede and follow the more focused studies that use enrichment cultures to test and verify particular hypotheses. Figure 1 represents this combination of approaches, and our case studies show how successful it can be to implement a combination of metagenomic strategies. But how is this methodological interplay connected to our starting point of microbial infallibility?

Back to microbial infallibility

What does microbial infallibility do for metagenomic research, especially when combined with enrichment cultures? We suggest that microbial infallibility helps guide hypothesis-driven metagenomics and sometimes exploration-driven metagenomics via several general emphases. First of all, microbial infallibility expectations provide a general theoretical framework for metagenomic investigations, and this more hypothesis-driven approach is a timely antidote to some of the exploratory data-driven excesses of metagenomic inventory studies. In particular, microbial infallibility guides enrichment specifications by setting theoretical expectations about how organisms might take energetic advantage of certain environments. Such expectations emphasize function and environment rather than taxa and genes for their own sake; they also foreground specific pathways and partnerships rather than whole communities, and this focus can make complex ecological situations more tractable. Especially when combined with enrichment culturing, microbial infallibility permits predictive testing of environmental hypotheses, which in turn allows generalizations about the living world that may ultimately tell us more about life in general (its nature and limits).

What is the relevance of that last point? If researchers are concerned with discovering life in obscure and extreme environments, or even elsewhere than on Earth, metabolic possibilities are a more productive line of theorizing than expecting certain taxa or biomarker molecules (highly contingent biological outcomes). Calculations about energy generation within defined chemistries show 'what is possible in nature and what is not' (Oren 2015, p. 4). Microbial infallibility is simply a restatement of such possibility in positive terms (i.e. living systems *should* take energetic advantage of these conditions). Metagenomics can then be used to investigate Earth-based niches (that may act as analogues for extraterrestrial environments) and reveal whether theoretical possibilities have been actualized, or how those expectations need to be modified.

For some researchers, the infallibility view can take a very strong form, postulating that life is an inevitable system that takes advantage of energetic imbalances. For example,

> 'The continuous generation of free energy abiotically may have forced life into existence to alleviate free energy stresses. This would imply that life *had to emerge* on Earth and on any similar planet' (Morowitz and Smith 2007, p. 51 slightly paraphrased).

This sort of view is the most hard-core and encompassing form of microbial infallibility that can be articulated, and is probably too strong for most readers, especially because the 'inevitability' is more a speculation than a workable hypothesis. There is no compelling reason to think these stresses would not be alleviated in the same abiotic manner in which they came into existence. Morowitz and Smith's very strong view is a universal expansion of 'everything is everywhere' that the Dutch founders of microbial ecology would probably not have considered. Weaker versions of microbial infallibility are likely to be more defensible, and more often made part of everyday research.

From this more modest perspective, microbial infallibility is not a theory or principle: it is a heuristic – an investigative strategy that guides enquiry, especially in complex situations, rather than providing laws or definitive answers. But the infallibility heuristic points to theory (thermodynamics and kinetics) that can generate useful hypotheses that – with carefully designed enrichment cultures – can be tested, even if some

thermodynamic scenarios would require hitherto undetected biochemical pathways to exist (see Kuypers et al. 2018). Environmental depletions or surpluses of particular chemicals are an obvious starting point for more focused hypothesis generation. And since chemistry is universal (i.e. beyond this planet), this means microbial infallibility can guide astrobiology too.

Microbial infallibility as a research heuristic explores the energetic limits to life. It also acknowledges the ingenuity of life and lifeforms in finding ways to circumvent known limits. Instances of such inventiveness include sequestering hydrazine, or working around challenging energetic situations by forming syntrophies (e.g. anaerobic methane oxidizers and sulfate reducers). The heuristic of microbial infallibility thus navigates between energetic universals and biological contingencies. For example, despite the energetic permissibility of Broda's first prediction (an organism using ammonium as an electron donor in anoxygenic phototrophy and fixation of CO_2), or Broda-like predictions for sulfur comproportionating organisms (Amend et al. 2020), the relevant organisms might simply never have come into existence (or no longer exist) because of the contingency of evolution. Moreover, kinetic barriers should be considered in addition to thermodynamic ones. This is best exemplified by the energetically costly process of N_2 fixation. Although there are theoretical N_2 reduction pathways that are exergonic, the kinetic stability of the nitrogen triple bond precludes organisms from using these pathways to conserve energy (Howard and Rees 1996; Oren 2015). This might demonstrate true recalcitrance of the molecule, and at least for any foreseeable evolutionary future, fallibility on the side of microorganisms.

Regardless of whether microbes turn out to be omnipotent or not, thinking in terms of microbial infallibility guides scientific inquiry by providing a perspective that narrows down complexity and increases predictive power. And in this era of data deluges, microbial infallibility is of demonstrated value in transforming metagenomics into more focused and hypothesis-driven enquiry.

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