Received: 25 January 2023 | Revised: 24 February 2023 | Accepted: 26 February 2023

DOI: 10.1111/mmi.15051

PERSPECTIVE

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# Perspective elucidating the physiology of a microbial cell: Neidhardt's Holy Grail

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**Funding information**
Deutsche Forschungsgemeinschaft and by the region of Saxony-Anhalt.

## Abstract

A living microbial cell represents a system of high complexity, integration, and extreme order. All processes within that cell interconvert free energy through a multitude of interconnected metabolic reactions that help to maintain the cell in a state of low entropy, which is a characteristic of all living systems. The study of macromolecular interactions outside this cellular environment yields valuable information about the molecular function of macromolecules but represents a system in comparative disorder. Consequently, care must always be taken in interpreting the information gleaned from such studies and must be compared with how the same macromolecules function in vivo, otherwise, discrepancies can arise. The importance of combining reductionist approaches with the study of whole-cell microbial physiology is discussed regarding the long-term aim of understanding how a cell functions in its entirety. This can only be achieved by the continued development of high-resolution structural and multi-omic technologies. It is only by studying the whole cell that we can ever hope to understand how living systems function.

**KEYWORDS**
energy conservation, metabolism, physiology, whole cells

## 1 | BACKGROUND

Life is currently considered to have evolved deep in the ancient Hadean ocean, around 3.8–4.2 billion years ago, most likely in alkaline hydrothermal vents (Martin et al., 2008). The mechanisms involved in the evolution of life are not currently understood. Nevertheless, the process must have been determined by the inherent inorganic chemistry of the early earth (Williams & Rickaby, 2012), with dissolved $CO_2$, methane, and formic acid acting as possible carbon sources and with the reducing power to "fix" this carbon into organic material probably being supplied by $H_2$ oxidation (Branscomb & Russell, 2018b). When the huge variety of microbial life (valid for all life forms) currently extant on earth is considered, it can be viewed as a "continuum"; cells do not spontaneously "spring into life out of thin air"—life begets life. This continuity of living forms from life's emergence, by whatever mechanism this might have involved, is supported by phylogenetic analyses that indicate a universal common ancestor for all life forms, underpinned by the unity of biochemistry (Thauer, 1997; Weiss et al., 2018); however, with possible caveats (Schada von Borzyskowski et al., 2020; Torres de Farias et al., 2021). The astounding facet of life is that it defies the second law of thermodynamics, namely, living cells continuously maintain a low entropy, or to adopt Schrödinger's evocative phrase, life sucks "*…orderliness from its environment…*"; in other words, living organisms need to harness negative entropy to maintain themselves (Schrödinger, 1944). Essentially, living systems delay the release of

entropy to their environment, and the way they do this is by continuously "driving" endergonic (thermodynamically "uphill") processes, in a highly specific manner, into a state far from equilibrium (disequilibrium) using coupled transformations (reactions) that are highly exergonic (Branscomb & Russell, 2013, 2018a). Microbial cells (indeed, every living cell) comprise myriad such endergonic/exergonic pairs (disequilibria = "free energy"), all of which are inextricably interlinked and have been since life evolved (Branscomb & Russell, 2018a). They are continuously in a stably maintained and highly organized non-equilibrium state; cells effectively represent self-generating autocatalytic systems (Kauffman, 2020; Wächtershäuser, 1992).

So, why am I stating this? The reason is that whole cells represent an inherently irreversible system, where enzymes function in supra-molecular complexes, in a highly ordered gel-like matrix, with protein concentrations in the range of 200–300 mg $mL^{-1}$ (Elowitz et al., 1999; Zimmerman & Trach, 1991). The flow of metabolites, transcription of mRNA, DNA replication, etc., are catalyzed by highly ordered complexes that are exquisitely allosterically controlled and defy mass-action chemistry, which always seeks equilibrium (maximum entropy) (Branscomb & Russell, 2018a). In contrast, when we study enzymes, for example in cell extracts, or the purified state, in the absence of the cellular environment, there is no longer any order—"chaos" reigns supreme. While tremendous advances in microbial biochemistry and physiology have been made (and will continue to be made) by studying macromolecular functions in vitro, there remains a caveat, which was formulated already in the 1950s by Aleksandr Oparin "*… when we destroy mechanically the integrity of the living protoplasm, as by grinding it finely… the mixture thus obtained contains all of the enzymes which were present also in the living cells and all the substances which were previously acted upon in the course of biological processes. Nevertheless, the mixture no longer reproduces the chemical transformations observed in the living cell because it lacks a definite physio-chemical organization.*" (cited in Branscomb & Russell, 2018a). Thus, great care must be taken when drawing conclusions about the highly ordered cellular state based on the study of highly disordered cell extracts or protein solutions. Oparin already recognized that no matter how concentrated we make a cell extract after destroying cellular integrity, and no matter how we might try subsequently to energize it, it is impossible to imbue that extract with "life" (Oparin & Morgulis, 1953). The reasons why such a "Frankensteinian reanimation" of dead matter can never occur have been cogently and eloquently formulated (Branscomb & Russell, 2018a, 2018b). The point being made is that, despite the great import of what is gained by such studies, there is a very significant difference between how enzymes, or enzyme complexes, function in vitro compared with how they function within the cellular context, or in vivo; we are missing something very important. This was already made clear during the first half of the 20th century when many advances in our understanding of Microbiology were achieved by scientists working with whole cells rather than with cell extracts (see, e.g., Stephenson & Stickland, 1932). We as scientists have lost track somewhat of the importance of studying whole cells and in this perspective, I will attempt to highlight a few examples of how in vivo studies often deliver experimental results that are either unexpected or even differ significantly, when compared to when the experiments are performed in essentially weak protein solutions (extracts) that completely lack order. Many research groups are already implementing such in vivo approaches, particularly when studying the "uncultured microbial majority" (Colman et al., 2017; Hoehler & Jørgenson, 2013; Lee et al., 2020), and the tools they are developing and using in these approaches will prove crucial in future research in this direction. Realignment of our experimental approaches, with a stronger focus on the study of single bacterial or archaeal cells, and combining this information with what is learned using classical reductionist approaches, might be the route to understanding how cellular physiology, and perhaps even how "life" itself, really works. This was also recognized by Frederick Neidhardt and was the premise behind the "*E. coli* and *Salmonella* bible": Neidhardt sought "*to envision the solving of a cell, i.e., understanding one cell sufficiently well to permit a detailed mathematical model of it*" (Neidhardt, 1996).

## 2 | THE IMPORTANCE OF PROTEIN INTERACTIONS IN VIVO

Formic acid is an excellent electron donor for many anaerobic microorganisms. This is also the case for *Escherichia coli*, which can use its conjugate base, formate, as an electron source for respiration with oxygen or nitrate as an acceptor, or during fermentation, where it is imported into the cell by the formate-nitrite transporter channel protein, FocA (Kammel et al., 2022). In the cytoplasm, formic acid is disproportionated to $CO_2$ and $H_2$ by the membrane-associated formate hydrogenlyase (FHL) complex (Peters & Sargent, 2023; Steinhilper et al., 2022). The benefits to the cell of taking up formate are two-fold: first, if formic acid is imported into the cell, this helps offset acidification of the cell's immediate environment (Metcalfe et al., 2022); second, if formate, rather than formic acid, is taken up and subsequently formate plus a cytoplasmic proton are converted into gaseous $H_2$ plus $CO_2$ by the FHL complex (Peters & Sargent, 2023), then this helps offset acidification of the cytoplasm and, additionally, potentially contributes to the establishment of an ion (proton) gradient (Kammel et al., 2022). If the complex I-related FHL complex can also pump a proton across the membrane during this reaction, all the better for the cell; however, it is currently still debated whether this latter step occurs (Peters & Sargent, 2023). The archeon *Thermococcus onnurineus* grows by formate-driven $H_2$ production and uses a version of the FHL complex along with a FocA homolog to achieve this; however, the precise mechanistic details of how it grows at this apparent thermodynamic "energy limit" remain to be resolved (Kim et al., 2010).

The critical step in formate-driven $H_2$ production is the uptake of formate or formic acid by FocA, which in *E. coli* whole-cell studies is coupled to FHL complex activity (Beyer et al., 2013); if FHL is inactivated, formate remains outside the cell. FocA specifically translocates formate bidirectionally in whole cells (Kammel et al., 2022), but in vitro, FocA can translocate a variety of small monovalent

anions (Lü, Du, Schwarzer, et al., 2012). Moreover, an *N*-terminally truncated version of the FocA protein can translocate formate unimpeded in vitro (Wang et al., 2009), yet it fails to translocate formate in cells (Kammel et al., 2021). This is because it requires as interaction partner pyruvate formate-lyase, which binds to the cytoplasmically oriented *N*-terminal domain of FocA to "gate" the channel in vivo (Doberenz et al., 2014; Kammel et al., 2021, 2022). Thus, while the importance of the in vitro studies in providing key structural and biophysical information regarding FocA and its many orthologues in microorganisms (Lü et al., 2011, Lü; Schwarzer, Du, et al., 2012; Lyu et al., 2021; Waight et al., 2010; Wang et al., 2009) are pivotal and unquestioned, the extent to which the data relate to in vivo function must be interpreted with caution.

## 3 | WHOLE CELLS AND THE STUDY OF SUBSTRATE UTILIZATION

Some 40 years ago, the study of atmospheric trace gases revealed that particularly $H_2$ (~53 part per billion by volume—ppbv), CO (90 ppbv), and $CH_4$ (~1860 ppbv) are oxidized by soil ecosystems (reviewed in Conrad, 1996). Initially, these oxidation processes were considered to be both abiotic (cell-free) and through the activity of microorganisms. This important field has burgeoned in the last few years, especially in light of advances made in phylo(meta)genomics, and it has become increasingly clear that a broad range of bacterial and archaeal phyla are responsible for trace gas oxidation (Greening & Grinter, 2022). These microorganisms appear to have high-affinity enzyme systems to allow them to use these limited resources to supplement their energy budget. It has been estimated that more than 75% of abiogenically and biogenically produced $H_2$ is recovered from the atmosphere by bacteria and archaea (Conrad, 1996). Indeed, it appears from an analysis of diverse soil samples analyzed across the globe that trace levels of $H_2$ are scavenged by numerous bacterial phyla (Greening & Grinter, 2022). Remarkably, oligotrophic antarctic soils reveal a high abundance of $H_2$-consuming bacterial species (Ortiz et al., 2021), and the recent discovery of *Methylocapsa gorgona* (Tveit et al., 2019, 2021) as the first species recognized to "grow on air," by mixotrophically oxidizing $H_2$, CO, and $CH_4$ to fix $CO_2$ strengthens the premise that many bacterial species probably can use trace gases either to supplement their energetic needs for maintenance under nutrient limitation, or for growth (Greening & Grinter, 2022). Moreover, understanding how these processes function thermodynamically might help explain the mechanisms used by the dormant microbial majority in the deep biosphere to persist for hundreds, even thousands of years (Hoehler & Jørgenson, 2013).

The oxidation of these trace gases requires enzyme complexes with significantly higher affinities (apparent $K_m$, < 150 nM) than those exhibited by well-characterized, low-affinity enzyme complexes (apparent $K_m$, > 500 nM) (reviewed in Greening & Grinter, 2022). For example, in the case of $H_2$ oxidation, the bacterial species encode high-affinity [NiFe]-hydrogenases, belonging to either group 1h, 1f, 1l, and 2a (Hhy, Hyo, Hyl, and Huc, respectively) hydrogenases. These enzyme classes all show apparent insensitivity toward oxygen, which typically inactivates [NiFe]-hydrogenases (Lubitz et al., 2014). Moreover, these enzymes appear to be synthesized during "dormancy", which can adopt different physiological states, including dauer forms. For example, synthesis of an Hhy hydrogenase is induced specifically in spores of *Streptomyces avermitilis* and contributes to viability (Constant et al., 2010; Liot & Constant, 2016), while its non-sporulating relative, *Mycobacterium smegmatis*, adopts a similar strategy (Greening et al., 2014) and can likely couple $H_2$-oxidation to $NO_3^-$ as well as $O_2$ reduction.

Whole-cell studies with several bacterial species demonstrate a high affinity for all three trace gases, and high rates of whole-cell oxidation of $H_2$ and CO suggest that, at a minimum, maintenance energy needs can be met for the bacteria in these ecosystems (reviewed in Greening & Grinter, 2022). Notably, however, the only high-affinity Hhy hydrogenase isolated and characterized to date from *Methyacidiphilum fumariolicum* (Schmitz et al., 2020) fails to oxidize atmospheric $H_2$ concentrations, yet whole cells and membrane fractions do achieve this. There have been no reports yet of in vitro analyses performed with high-affinity carbon monoxide dehydrogenases or methane-oxidizing enzymes. The findings reported for the high-affinity Hhy hydrogenase from *M. fumariolicum* indicate that separation of the enzyme from the environment of the cell (or membrane) is clearly detrimental to activity and possibly indicates that direct coupling of the enzyme complex with the rest of the respiratory chain is crucial to allow the manifestation of $H_2$ oxidation.

## 4 | FUTURE APPROACHES TO UNDERSTANDING WHOLE-CELL PHYSIOLOGY

Adopting a multidisciplinary approach to studying bacterial and archaeal physiology will, in the long term, pay dividends. Many research groups are adopting methods to study bacteria and archaea both in vivo and in situ, also at the single-cell level. Molecular and evolutionary ecophysiologists are actively developing tools to advance our knowledge of the uncultivated microbial majority (Ge et al., 2022; Hoehler & Jørgenson, 2013; Lee et al., 2020). In particular, methods combining stable-isotope labeling, high-resolution fluorescence microscopy incorporating Raman spectroscopy techniques, and nanoscale secondary ion mass spectrometry allow the determination of nutrient utilization by single cells (Endesfelder, 2019; Ge et al., 2022; Lee et al., 2020; Taylor, 2019). Combining these methods with high-resolution multi-omics approaches, together with analysis of the structural biology of large enzyme complexes by coupling chemical cross-linking with cryo-electron microscopy (Piersimoni et al., 2022), and whole-cell cryo-electron tomography (cryo-ET) (Baumeister, 2022; Oikonomou & Jensen, 2017) will ultimately deliver new, and likely unexpected, insights into how cells function. Continual development of the methods in these fields will push the envelope of structural and metabolic high-resolution to reveal the physiology of the single cell.

Recent research findings that highlight the use of these multi-disciplinary approaches are already delivering remarkable new data. The recent enrichment and first description of the actin-based cellular architecture of the Asgard archaeon *Candidatus Lokiarcheon ossiferum* (Rodrigues-Oliveira et al., 2023), the closest archaeal relative of eukaryotes, was achieved by combining classical microbiology and phylogenomics with high-resolution fluorescence microscopy and cryo-ET. These studies also identified other complex surface and cytoplasmic structures, many of which may be related to the numerous eukaryotic signature proteins whose encoding genes are present on the genome of these archaea.

An example of the deployment of high-resolution and exquisitely detailed multi-omics approaches combined with physiological studies has recently revealed the complexity underlying the aromatic compound catabolic network of *Aromatoleum aromaticum* EbN1, a specialist in the degradation of recalcitrant hydrocarbons (Becker et al., 2022). A new, previously unexpected feature of the regulation of these catabolic pathways has shown that a lack of correlation between the transcriptome and proteome often occurs. This has the consequence that protein abundance is significantly higher than the correspondingly low levels of cognate mRNA transcripts and occasionally vice versa. The future overlaying of the structural identification of the enzyme complexes responsible for these degradation pathways using single cells with the profiles of metabolic intermediates of each pathway could potentially reveal live-action metabolism.

## 5 | POTENTIAL FUTURE SCIENTIFIC GAINS

The two examples of recent scientific advances mentioned above highlight the potential new insights to be gained by adopting high-resolution multi-disciplinary approaches. However, perhaps two of the most fascinating aspects of ultimately achieving a precise definition of the physiology of a single microbial cell will be identifying the functions of the numerous (~50% for *E. coli*) gene products of unknown function, and providing a description of how the bioenergetics of non-growing, dormant cells, and spores functions; spores are metabolically active but at a very low rate.

A recent study that took a novel whole-cell screening approach to look for *E. coli* mutants showing changes in $Ca^{2+}$-transients identified a large gene set linking $Ca^{2+}$-signaling to mechanosensation (Luder et al., 2021). As well as revealing potential new functions for known genes, numerous genes whose products have unknown functions were also identified. Moreover, the findings also provided a potential, although currently poorly understood, link to the cell's ion gradients, suggesting that *E. coli* might be "touch-sensitive" to its environment (Luder et al., 2021). This example underscores the benefits to a research field when scientists from other disciplines add their alternative perspectives. They enter the fray without any preconceptions (see e.g., Schrödinger, 1944).

This brings me to what, in my opinion, is a second pressing future issue, namely to attempt to elucidate the bioenergetics of dormancy. Considering that the vast majority of microbial cells in oligotrophic soils, or deep-sea sediments are in a dormant state (Hoehler & Jørgenson, 2013), how do they adapt their metabolism in order simply to "tick over" and survive, sometimes for thousands of years, barely growing, if at all? What mechanisms are used to minimize leakage of ion gradients over time? Endospore formation is likely to be one such evolutionary adaptation to deal with this physiological state, but employing a different ion gradient, e.g., using sodium, as many archaea do, especially when growing at high temperatures, may also result in more efficient ion gradient maintenance (Lane & Martin, 2012), thus aiding energy conservation. The attritional state, i.e., simply surviving, may well be the norm for many microorganisms and might reflect how bacteria and archaea evolved in the first place. Gaining a better understanding of this state is also highly relevant to elucidating pathogenesis mechanisms, including persistence (Greening & Grinter, 2022), as well as aiding the search for life on other planets (Colman et al., 2017).

It is conceivable that some of the roughly 50% of genes whose products have unknown functions may have a role in aiding the lowering of metabolic rate. Recent studies with *Bacillus* endospores have highlighted the importance of controlling ion gradients, particularly potassium, in inducing exit from dormancy (Kikuchi et al., 2022). Yet, despite the false claim that spores and other cells are frequently referred to as 'physiologically inactive' if they can be reanimated then they are far from metabolically "inactive" and have not achieved maximal entropy: after all, dead remains dead. If we can begin to understand these bioenergetic enigmas by combining reductionist with whole-cell approaches, then we have a chance of understanding how life might have evolved in the first place. As Schaechter and Neidhardt presciently stated, "*Until we understand thoroughly the growth [physiology] of one cell, we shall be handicapped in imagining the nature of our understanding of any cell.*" (Schaechter & Neidhardt, 1987). Ultimately, metabolism drives all living systems and this is what we must strive to understand in its entirety (de Lorenzo, 2015).

### AUTHOR CONTRIBUTIONS

RGS conceived and wrote the study.

### ACKNOWLEDGMENTS

I would like to thank Michelle Kammel for discussion. Work in the author's laboratory is supported by the Deutsche Forschungsgemeinschaft and by the region of Saxony-Anhalt. Open Access funding enabled and organized by Projekt DEAL.

### CONFLICT OF INTEREST STATEMENT

The author declares that there is no conflict of interest to report.

### DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

### ETHICS STATEMENT

The author confims that he has adhered to all ethical considerations during the preparation and writing of this text. The author

guarantees that no part of this manuscript was written using Artifical Intelligence tools and contains no Articifial Intelligence Generated Content.

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**How to cite this article:** Sawers, R. G. (2023). Perspective elucidating the physiology of a microbial cell: Neidhardt's Holy Grail. *Molecular Microbiology*, *00*, 1–6. https://doi.org/10.1111/mmi.15051