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Opinion

Respiration-induced biofilm formation as a driver for bacterial niche colonization

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Depending on their physiology and metabolism, bacteria can carry out diverse redox processes for energy acquisition, which facilitates adaptation to environmental or host-associated niches. Of these processes, respiration, using oxygen or alternative terminal electron acceptors, is energetically the most favorable in heterotrophic bacteria. The biofilm lifestyle, a coordinated multicellular behavior, is ubiquitous in bacteria and is regulated by a variety of intrinsic and extrinsic cues. Respiration of distinct electron acceptors has been shown to induce biofilm formation or dispersal. The notion of biofilm formation regulation by electron acceptor availability and respiration has often been considered species-specific. However, recent evidence suggests that this phenomenon can be strain-specific, even in strains sharing the same functional respiratory pathways, thereby implying subtle regulatory mechanisms. On this basis, I argue that induction of biofilm formation by sensing and respiration of electron acceptors might direct subgroups of redox-specialized strains to occupy certain niches. A palette of respiration and electron-transfer-mediated microbial social interactions within biofilms may broaden ecological opportunities. The strain specificity of this phenomenon represents an important opportunity to identify key molecular mechanisms and their ecophysiological significance, which in turn may lay the ground for applications in areas ranging from biotechnology to the prevention of antimicrobial resistance.

Highlights

The biofilm lifestyle is the prevalent prokaryotic mode of life in numerous habitats and represents a niche colonization mechanism subjected to a swift regulation by external and internal cues. Respiration is a ubiquitous form of energy acquisition. Metabolic resources driving competition for a niche include electron acceptors used for respiration.

Growing evidence from diverse bacterial species suggests intimate links between respiration and biofilm turnover. This ubiquity could imply a potential universal nature of this phenomenon across the kingdom Bacteria. Recent evidence shows that respiration-induced promotion or dispersal of biofilms can be strain-specific even if the same respiration pathways are conserved, functional, and expressed by the individual strains.

Respiration-induced biofilm formation might aid the occupation of distinct niches by redox-specialized species or strains within the same species, potentially contributing to bacterial speciation, and opening up for a palette of yet virtually unexplored social microbial interactions.

Understanding respiration-induced biofilm formation mechanisms will provide novel insights into microbial ecology, ranging from bacterial community structure and composition in stratified environments to host colonization by bacterial pathogens or commensals. Targeting or engineering such mechanisms may enable an array of applications in biomedicine or biotechnology and lay the ground to new approaches for fighting antibiotic resistance.

Respiration-induced biofilm formation and dispersal in the context of bacterial niche occupation

A hallmark of life is energy conservation through redox reactions. This paradigm is the essence of bacterial **energy metabolism** (see Glossary) and **cellular respiration** (Box 1). Facultative anaerobic bacteria employ molecular oxygen or alternative electron acceptors (AEAs) as the terminal sink for the electrons shuttled across the **electron transport chain**. This physiological versatility allows facultative anaerobes to thrive in oxygen-rich as well as oxygen-depleted environments. A primary strategy to occupy an ecological niche is through the formation of a **biofilm**. Benefits of biofilm formation include protection against physical and chemical stressors, environmental persistence, and a more efficient acquisition and use of nutrients. These collective advantages promote the ubiquity and resilience of this **lifestyle** in nature. Indeed, recent estimates highlight the biofilm lifestyle as the prevalent prokaryotic mode of life in numerous ecological niches [1]. Biofilms can occur in environments with variable levels of oxygen or AEAs. At the same time, stratified multispecies communities within biofilms are exposed to intrinsic oxygen and nutrient gradients that generate distinct local microenvironments, thereby imposing energy acquisition to occur through a certain set of reactions.

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Box 1. Energy metabolism

Energy metabolism is a hallmark of cellular life. Generation of ATP occurs most efficiently through the establishment of a redox potential gradient between an electron donor and acceptor (see Figure 2A in the main text). The most efficient means for energy generation are photosynthesis and either aerobic or anaerobic respiration. Bacterial respiratory chains are remarkably flexible and can employ diverse combinations of electron donors and acceptors, even simultaneously. In the absence of oxygen or suitable AEAs, fermentation generates low ATP yields using an endogenous electron acceptor, most frequently pyruvate, through substrate-level phosphorylation. Fermentation waste-products include carbon dioxide, hydrogen gas, and a diversity of organic compounds, such as ethanol or carboxylic acids, with industrial and environmental significance.

Respiration generates ATP through membrane-associated ATP synthases. Recycling of reduction equivalents creates energy through electron transport at the (inner) membrane, ultimately building up a proton-motive force required for ATP synthesis. From an energetic standpoint, aerobic respiration, which uses oxygen as terminal electron acceptor, is the most efficient mode of ATP generation. Facultative and obligate anaerobes employ anaerobic respiration using inorganic compounds (e.g., NO_3^-, Fe^{3+}, SO_4^{2-}) or organic compounds [e.g., trimethylamine-*N*-oxide (TMAO), dimethylsulfoxide (DMSO)] as final electron acceptors. Anaerobic respiration has a wide impact on the global nitrogen and sulfur cycles. Denitrification, the reduction of inorganic nitrate, results in the production of molecular nitrogen, a gas that represents 78% of the atmosphere of planet Earth. The biogeochemical cycle of sulfur includes reduction of inorganic sulfate and sulfur intermediates to H_2S, and ultimately to elemental sulfur. Hydrogen sulfide feeds anoxygenic photosynthesis by certain groups of phototrophs, such as green sulfur bacteria.

Organic electron acceptors of broad ecological relevance are molecules such as TMAO and DMSO. TMAO is an osmolyte of marine animals. Trimethylamine, the product of TMAO reduction, constitutes an important seafood spoilage metabolite. DMSO is generated by bacterial and photochemical oxidation of dimethyl sulfide, or secreted by organisms such as algae and phytoplankton. In the atmosphere, DMSO molecules act as cloud condensation nuclei, leading to an increase in albedo, a phenomenon with major repercussion for global climate.

Biogeochemical cycles are often intertwined. For example, metal ions like Fe(III) or Mn(IV) also play significant biological and geochemical roles, and their natural cycles overlap closely with those of sulfur and nitrogen.

Two limiting resources drive microbial competition for a niche, namely, space, which provides the physical environment and mechanical support, and nutrients, which are essential for energy acquisition [2]. Thus, in essence, microbial niche occupation involves gaining control over the metabolic resources available at a certain habitat, which include electron acceptors whose utilization may provide a **fitness** advantage. Bacteria employ a plethora of strategies for niche occupation, largely marked by a constant warfare with other microbes [3,4]. In terms of lifestyle, the decision for the individual bacterial cell is reduced to essentially two possibilities (Figure 1A): either **planktonic** (free-living) or sessile (biofilm-associated).

In this opinion article I summarize data that suggest respiration to be an active, ubiquitous driver for biofilm formation and niche occupation in bacteria (Box 2). I provide evidence showing that biofilm formation, or shifts in biofilm amount, are deeply intertwined with respiration of distinct electron acceptors, and that these links can be species or strain-specific. That is, respiration can induce biofilm formation or dispersal, a process that I refer to as 'respiration-induced biofilm formation' (RIBF) or 'respiration-induced biofilm dispersal' (RIBD), respectively. While examples of both phenomena are presented, the main focus of this opinion piece is on RIBF because of its implication in surface colonization.

Importantly, the strain-specificity of RIBF and RIBD strongly points to the existence of subgroups of strains within the same species that I postulate might be directed to occupy rather specific niches, aided by subtle regulatory mechanisms that go beyond the mere metabolic capacity to use a given energy resource and therefore energy acquisition per se. Thus, as electron acceptor-specific biofilm formation can occur in strains that otherwise share the same functional respiratory pathways, I discuss mechanisms and additional physiological, genomic, and gene-expression characteristics that may aid niche colonization by specialized strains, potentially contributing to bacterial

Box 2. Is RIBF universal?

The iron–sulfur world hypothesis by Günter Wächtershäuser [21] already postulated the first cells – and the first energy metabolism – to have evolved from ancestral 'surface metabolists', primeval acellular organisms anionically bound to mineral surfaces containing iron, nickel, and sulfur, which catalyzed the synthesis of the precursor organic compounds needed for life. Fe-S clusters mediate electron transfer reactions. Fe-S cluster-containing proteins are universal in all branches of life and participate in diverse cellular processes, including the electron transport chain and respiration. Irrespective of how the first cells appeared and evolved, it is clear that a surface-associated, biofilm mode of life is deeply rooted into the biology of bacteria, and that this lifestyle has tight links with energy metabolism. Indeed, RIBF extends phylogenetically and seems to be widespread in bacteria. In colony biofilms of the gammaproteobacterial species *E. coli* and *Salmonella*, the so-called *rdar* (red, dry, and rough) morphotype on Congo Red-supplemented agar is characterized by the production of biofilm extracellular matrix components, primarily the exopolysaccharide cellulose and fibril-forming amyloid curli fimbriae. Strains of different *E. coli* pathotypes, including uropathogenic [22] and enterotoxigenic strains (Martín-Rodríguez *et al.*, unpublished), exhibit distinct colony biofilm morphotypes on nitrate-supplemented agar compared with nitrate-free medium. Thereby, certain strains downregulate *rdar* biofilm formation upon nitrate addition whereas a subset of strains exhibit exacerbated *rdar* morphotypes. The alteration in colony morphotypes is respiration-mediated [22] and likely represents alternative lifestyle adaptation mechanisms to electron acceptor abundance or electron acceptor-induced stress. Research with *Pseudomonas aeruginosa* PA14 colony biofilms supports this notion [23,24]. *P. aeruginosa* offers indeed interesting insights into the intertwined metabolic links between respiration, redox homeostasis, and biofilm morphogenesis. In *P. aeruginosa* PA14, an orphan *cbb3*-type cytochrome *c* oxidase has been shown to support cell survival within biofilms via reduction of oxygen and the electron shuttle phenazine [25]. Phenazines in turn have been shown to modulate PA14 intracellular c-di-GMP pools via the PAS-domain-containing c-di-GMP phosphodiesterase RmcA [26], thereby regulating the biosynthesis of the biofilm exopolysaccharide Pel. Biofilms are stratified communities in which cells have different accessibility to oxygen and AEAs depending on their location. Such a stratification leads to the existence of bacterial subpopulations with distinct gene expression programs. In biofilms of the uropathogenic *E. coli* (UPEC) strain UTI89, production of adhesive type 1 fimbriae is positively regulated by oxygen, and consequently these appendages are expressed by air-exposed cells [27]. Within colony biofilms, subpopulations of UTI89 cells express distinct levels of cytochrome *bd*, a proton-motive-force-generating terminal oxidase that positively contributes to biosynthesis of biofilm extracellular polymeric substances (EPSs) and colonization of organ niches during urinary tract infection [28].

Respiration control of a multicellular, biofilm-associated lifestyle has also been documented beyond *Gammaproteobacteria*. In the Gram-negative, facultative anaerobic betaproteobacterium *Neisseria gonorrhoeae*, an obligate human pathogen and causative agent of gonorrhea, respiration via the truncated AniA–NorB denitrification pathway was shown to be required for biofilm establishment [29]. In the Gram-positive-dominated phylum Firmicutes, respiration-mediated biofilm regulation has been reported for the species *Bacillus subtilis* [30,31] and *Staphylococcus aureus* [32,33], thereby showcasing the potential universality of this phenomenon. In most cases, though, strain-specificity of respiration-mediated biofilm formation has not been addressed and therefore the responses of model strains are often assumed to be representative of the entire species.

speciation. Finally, I present insight on how RIBF can become a target for novel therapeutic agents, or for the controlled modulation of beneficial biofilms in diverse contexts.

Respiration as a stimulator of planktonic lifestyles

A strategy to outcompete less-fit counterparts involves the use of the available resources to support planktonic population expansion (Figure 1A). For example, *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) strain IR715 elicits intestinal inflammation, thereby inducing an oxidative burst that results in tetrathionate production from gut sulfur pools. Locally produced tetrathionate acts as a terminal electron acceptor that boosts pathogen growth and facilitates its dissemination [5]. The generation of reactive oxygen and nitrogen species during colonic inflammation results in local production of nitrate, an AEA that triggers *S.* Typhimurium IR715 anaerobic metabolism and induces the expression of the *prpBCDE* operon enabling propionate breakdown [6]. Propionate is a short-chain fatty acid that causes toxicity and growth arrest in *S.* Typhimurium, so its degradation supports population expansion by the pathogen. Remarkably, parts of the *prpBCDE* operon are non-functional in extraintestinal *Salmonella* serovars, a signature of metabolic specialization [7]. Likewise, host-derived nitrate is used by diverse commensal *Escherichia coli* strains to outgrow the rest of the resident microbiota in the large intestine upon inflammation [8]. A recent study showed that nitrate induces biofilm dispersal in *S.* Typhimurium

Glossary

Biofilm (bacterial): a community of (bacterial) cells embedded in a self-produced extracellular matrix of heterogeneous composition, primarily proteins, exopolysaccharides, lipids, and extracellular DNA, that confers protection against environmental stressors. In nature, virtually all biofilms are multispecies and polymicrobial (i.e., they involve diverse species of bacteria and other microbes such as archaea, fungi, or microalgae).
c-di-GMP phosphodiesterase: an enzyme that hydrolyzes c-di-GMP through its catalytic EAL or HD-GYP domain.
Cellular respiration: a set of metabolic reactions aimed at generating energy from the oxidation of substrates involving a flow of electrons across the inner membrane. During respiration, the energy released from the oxidation of organic substrates (chemoorganotrophic bacteria) or inorganic substrates (chemolithotrophic bacteria) is used for the generation of a flow of electrons, which, through successive redox reactions in the inner membrane, feed a proton gradient that fuels an ATP synthase. The ultimate electron sink is a terminal electron acceptor, namely oxygen during aerobic respiration, or an alternative molecule (alternative electron acceptor) during anaerobic respiration.
Chemotaxis: migration of bacteria towards or away from effector molecules.
Cyclic di-GMP: a ubiquitous, almost universal dinucleotide second messenger in bacteria, known to regulate the lifestyle switch from planktonic to biofilm-associated and vice versa.
Cytochrome: a heme-containing, redox-active protein involved in electron transport.
Diguanylate cyclase: an enzyme that synthesizes c-di-GMP through its catalytic GGDEF domain.
Electron shuttle: organic molecules that act as electron carriers by undergoing reversible changes in their redox state.
Electron transport chain: proteins and organic molecules in the bacterial inner membrane that participate in redox reactions that translocate protons to the periplasm and ultimately shuttle electrons to a terminal electron acceptor.
Energy metabolism: a set of biochemical reactions devoted to energy

IR715 and increases motility, processes that may facilitate the spread of the pathogen in the inflamed gut [9]. In these examples, the ability of facultative anaerobic enterobacteria to use an array of AEAs represents a growth advantage over the native flora. Indeed, respiration of distinct electron acceptors can induce a lifestyle switch in cells already living within a biofilm. Despite an outstanding palette of anaerobic respiration pathways that may facilitate adaptation to hypoxic or anoxic niches, a sudden drop in oxygen tension acts as an acute biofilm dispersal cue for *Shewanella oneidensis* MR-1 [10]. Notably, such a dispersal was not recorded in response to electron donor depletion, irrespective of the presence of an AEA such as fumarate in the medium [10], showcasing its specificity to oxygen. The ecological significance of swift oxygen depletion as a biofilm dispersal cue in a freshwater sediment-native strain so well equipped for life without oxygen is intriguing.

Respiration, biofilm formation, and niche partitioning: lessons from *Shewanella*

A distinct strategy for niche occupation is the promotion of surface colonization through biofilm formation (Figure 1A). In this sense, recent research on *Shewanella* spp. offers novel insights into the intertwined links between energy metabolism and lifestyle regulation for niche colonization. *Shewanella* is a gammaproteobacterial genus globally distributed in marine and freshwater ecosystems, including sediments and host-associated. *In vitro* evidence from *Shewanella oneidensis* MR-1, an archetypal freshwater model strain, shows that early surface attachment minimizes competition and excludes more fit competitors from niche occupation: 'first come, first served' [11]. Experiments with *Vibrio cholerae* support this notion [12]. Work with the marine bacterium and occasional human pathogen *Shewanella algae* suggests that specialized strains can selectively use distinct electron acceptors for surface colonization, even if they share the same respiration pathways enabling their utilization [13]. *S. algae* and its close relative *Shewanella chilikensis* harbor two non-redundant periplasmic nitrate reductases involved in nitrate respiration that are largely conserved across strains. While nitrate reduction was observed in all isolates, only a subset of them exhibited increased biofilm formation upon nitrate supplementation. For certain strains, the opposite response, that is, reduced biofilm formation, was observed. Mutational analyses in combination with site-directed mutagenesis on the 4Fe-4S cluster of *S. algae* CECT 5071 nitrate reductase Nap-α showed the biofilm response to depend on catalytic activity [13]. A similar strain-dependent biofilm formation shift was observed upon supplementation of another electron acceptor, DMSO, whose respiration takes place extracellularly [13]. Both the nitrate and DMSO reductases are branches of a common electron transport chain rooted in the membrane-bound *c*-type **cytochrome** CymA. A subsequent transposon mutagenesis study identified additional respiration genes affecting different biofilm phenotypes in *S. algae* CECT 5071 [14]. Collectively, this implies intricate metabolic links between respiration and regulation of biofilm formation. It also points to the existence of specialized *S. algae* strain populations exhibiting increased surface colonization in experimental microcosm environments where electron acceptors are abundant, resembling natural ecosystems such as stratified water bodies and sediments, marine snow, or the intestinal tract of fish and aquatic invertebrates. It remains to be determined whether these strain-specific responses correlate with the occupation of distinct ecological niches by *S. algae* strains.

In the strongly stratified Baltic Sea, redox-specialized *Shewanella baltica* communities have been described across the oxic–anoxic interface [15,16]. Niche partitioning in *S. baltica* communities across the chemocline is favored by lateral gene transfer and recombination [17] resulting in gene gain and loss events involving respiration pathways. Thereby, *S. baltica* clade E strains from the suboxic zone are unable to employ the AEAs TMAO and thiosulfate, presumably because of lack of functionality or expression of the TMAO reductase [15,16]. Evidence of loss of DMSO reductase, concomitant with unaltered biofilm formation in the presence of DMSO, has

acquisition by the cell through ATP biosynthesis. Metabolic processes that lead to energy acquisition in bacteria include photosynthesis and respiration (oxidative phosphorylation) as well as fermentation (substrate-level phosphorylation).
Energy taxis: migration of bacteria towards environments with the optimal levels of a metabolic resource.
Fitness: in the scope of this article, bacterial ability to adjust their metabolism to adapt to the environmental conditions.
Lifestyle: bacterial mode of life. In the scope of this opinion piece, it is either free-living (planktonic) or multicellular (biofilm).
Planktonic: the lifestyle of individual, free-living bacteria, in contrast to sessile multicellularity (biofilm).

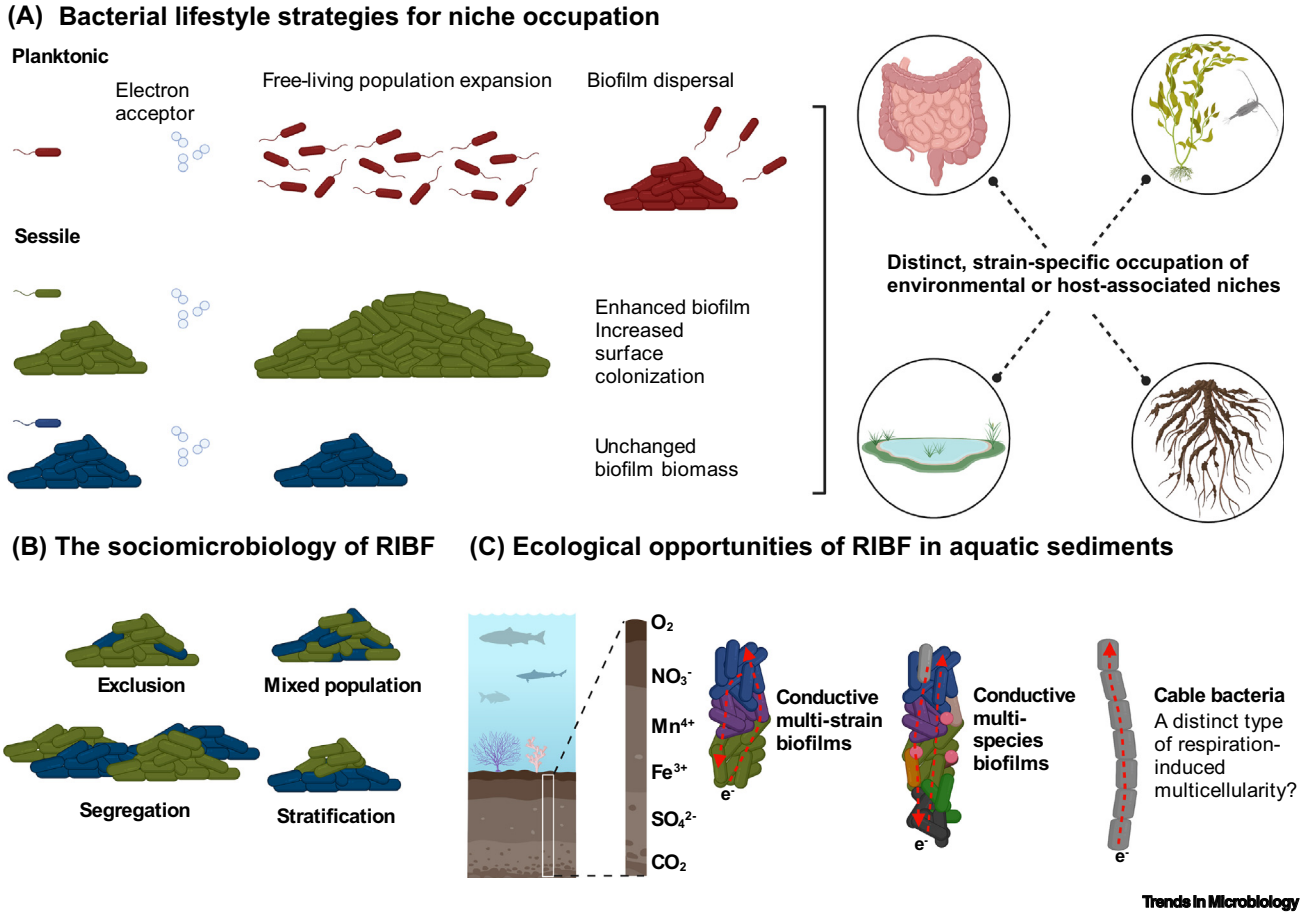

Figure 1. Bacterial strategies for niche occupation and interactions within biofilms. (A) Respiration of distinct electron acceptors (represented as bubbles), supports competitor exclusion by certain bacterial species or strains through planktonic population expansion. Induction of a planktonic lifestyle in biofilm-growing cells, that is, biofilm dispersal, is also documented. An alternative strategy for niche occupation is surface colonization via biofilm formation. Thereby, specialized species or strains selectively respond to the abundance and utilization of distinct electron acceptors with shifts in biofilm formation. Strain-specific differences may direct subgroups of strains to occupy certain environmental or host-associated niches. (B) Strain-specific responses might allow multiple potential antagonistic or cooperative social interactions in mixed biofilms, determining biofilm community structure. (C) Respiration-induced biofilm formation is most relevant in environments with a variety of electron acceptors, such as the inherently stratified sedimental milieu. Ecological opportunities include the formation of conductive biofilms formed by distinct strains within the same species occupying redox-specialized niches, as well as multispecies biofilms, in which a flow of electrons can be established in the multicellular community. Cable bacteria represent a peculiar type of multicellularity in which the individual cells play specialized redox roles according to their spatial position. Abbreviation: RIBF, respiration-induced biofilm formation.

also been documented in *S. algae* [13], as well as horizontal acquisition of nitrite reductases from distinct *Shewanella* spp. [18], whose potential contribution to biofilm formation awaits further investigation. *S. baltica* clades representing different ecotypes are able to selectively use a distinct array of electron acceptors during anaerobic growth, which correlates with the expression levels of the corresponding reductases and distinct transcriptional landscapes including strain-specific genes [19]. While biofilm formation in relation to electron acceptor utilization has not been investigated in *S. baltica* strains, potential links with organic particle-associated lifestyles have been implied for some clades [15,19].

Marine bacteria inhabit a global ecosystem, the ocean, that is highly diverse at both the microscale and macroscale. Marine *Shewanella* species are ubiquitous, inhabiting oxygenated surface

waters but also anoxic deep-sea sediments. A recent study postulated that abyssal *Shewanella* spp. may have evolved from upper-ocean species through respiratory specialization, undergoing gene losses involving nitrate, DMSO, and TMAO respiration pathways [20]. While this hypothesis requires further validation, the collective evidence presented here suggests that respiration and redox specialization are active drivers in the diversification of *Shewanella* populations in distinct ecological niches.

Respiration and microbial social interactions in biofilms

Natural biofilms are mixed communities and a hotspot for microbial social interactions. I have pointed out that RIBF can represent a strain-specific trait to outcompete other strains or species by preventing their surface attachment. However, could it also be, under certain ecological contexts, a cooperation-promoting trait? Would the presence of electron acceptors on a substrate or in the medium lead to an exclusion of competitor strains by biofilm-responsive strains, or would distinct multistrain biofilm combinations emerge, such as mixed-strain populations, segregated-strain lineages, or layered niche-specialist stratification? (Figure 1B). Although there is a growing interest in the sociomicrobiology of biofilms [34], there is currently a gap in knowledge on respiration-mediated interactions. In mixed biofilms, electron carriers feeding certain bacterial respiration pathways could represent some sort of 'public good'. This notion is supported by experimental evidence that extracellular **electron shuttles** produced by wild-type *S. oneidensis* MR-1 cells are able to rescue the anaerobic growth of menaquinone biosynthesis-deficient mutants [35]. Being a potential public good, intercellular electron transfer could be exploited by non-producing cells, similar to siderophore cheating in *Pseudomonas* or *Vibrio* populations [36,37]. Mutualism between *E. coli* and *S. oneidensis* MR-1 cells in an experimental laboratory setup was proposed, putatively involving the utilization of flavins (organic electron shuttles produced by *S. oneidensis* MR-1) by *E. coli* (a non-producer organism) to facilitate respiration and mixed biofilm formation on electrodes [38]. In mixed biofilms of *S. aureus* and *Enterococcus faecalis*, commonly coexisting species in nosocomial biofilm infections, heme produced by *S. aureus* feeds *E. faecalis* aerobic metabolism, resulting in a dual-species biofilm upshift facilitated by *E. faecalis* gelatinase-mediated hydrolysis of conjugated heme [39]. *E. faecalis* lacks a Krebs cycle and is therefore unable to synthesize heme, thereby requiring exogenous activation of aerobic respiration. Interestingly, this interspecies association was shown to be strain-specific, largely depending on differences in aerobic respiration or heme uptake, among other factors, by *E. faecalis* strains [39].

Electron transfer within monospecies biofilms of *Geobacter* or *Shewanella* in association with the respiration of insoluble electron acceptors is well documented [40,41] and exemplifies a multicellular behavior oriented to maximize the collective benefits of environmental redox resources. Thereby, biofilms of a thickness of several micrometers are formed by members of these genera with electrons flowing from one cell to another through a transfer network involving outer membrane cytochromes, conductive cellular appendages (nanowires), electron shuttles, and biofilm EPSs [41,42]. Electron transfer may occur preferentially among planktonic or biofilm populations. Thus, electrical transfer is higher in *Shewanella loihica* PV-4 biofilm cells than among their free-living counterparts [43]. By contrast, electrical transfer in *S. oneidensis* MR-1 is favored in planktonic cell populations [43,44]. The higher number of *c*-type cytochrome gene content in the metal reductase-containing locus of PV-4 when compared with that of MR-1 could be a reason contributing to this different behavior [43]. Such evidence supports the notion of specialized respiration-mediated lifestyle decisions by species or strains within the same species for niche colonization, in this case potentially determined by distinct genetic content.

Extracellular electron transfer strategies are not exclusive to mineral-respiring bacteria and have been recently documented in Gram-positive gut bacteria as well, like *E. faecalis* or *Listeria*

monocytogenes, with evidence for similar electron shuttle-mediated pathways being present in other members of the phylum Firmicutes [45,46]. The collective benefits of electron transfer in the biofilm community go beyond species borders and there is now clear evidence for direct or electron-shuttle-mediated interspecies electron transfer [47–49] (Figure 1C). Extracellular electron transfer generally occurs within a relatively narrow spatial range. Long-distance electron transfer had been identified in Gram-negative bacteria and recently, in the Gram-positive, filamentous unicellular bacterium *Lysinibacillus varians* strain GY32 [50]. GY32 was shown to perform long-distance, bidirectional extracellular electron transfer in biofilms via the production of nanowire-like appendages and *c*-type cytochromes [50]. Collectively, these findings suggest that redox interactions between coexisting microbes might be more common and widespread in nature than previously thought.

Cable bacteria represent an insightful case from an electrical cooperation standpoint. Abundant in marine and freshwater sediments globally, cable bacteria of the family Desulfobulbaceae form multicellular filaments capable of transferring electrons over centimeter distances, coupling sulfide oxidation in the deeper layers of the sediments with oxygen reduction close to the surface [51] (Figure 1C). This represents a rather remarkable form of multicellularity. The nature of the conductive cellular structures enabling cell-to-cell electron transfer in cable bacteria is still unknown, although genomic and experimental evidence points to *c*-type cytochromes as primary candidates [52], functionally analogous (but evolutionarily different) to *c*-type cytochrome-mediated cell-to-cell electron transfer in *Geobacter* or *Shewanella*. Notably, consistent with their strict anaerobe ancestry, only cells in the 'anodic' side of the filament performing sulfide oxidation can benefit from energy acquisition to actively divide, whereas cells in the 'cathodic' zone performing oxygen reduction cannot couple this reaction with biomass generation [52,53]. To compensate for the metabolic costs of oxygen exposure, which include coping with oxidative stress and an increased pH, experimental evidence suggests that cells within the filaments occupy the oxic area in turns [53]. This peculiar type of multicellular association has certain parallelisms with strain-specific RIBF, whereby distinct strains or subpopulations of the same strain are postulated to colonize niches in function of their redox specialization, potentially leading to the establishment of cooperative associations.

Mechanistic insights into RIBF

Promotion or dispersal of biofilms is a critical lifestyle decision that requires a swift integration of environmental stimuli coupled with genetic and physiological reprogramming. Mediators and mechanisms of RIBF are diverse and likely variable across phylogroups. It is not in the scope of this opinion piece to provide a comprehensive review on this subject. In the following, an overview of present knowledge is provided, with emphasis on interaction of respiratory components with diverse signaling circuits controlling biofilm formation, and additional mechanisms are proposed based on current evidence (Figure 2B, Key figure).

Genomic content

The irruption of second- and third-generation sequencing technologies has facilitated genomic analyses to unprecedented levels of throughput and detail. Sequence divergence including indels, or nucleotide polymorphisms leading to conservative or non-conservative amino acid replacements in respiratory reductases, signal transduction systems, or regulatory and biosynthetic pathways towards biofilm formation, may affect the functionality or responsiveness of proteins or pathways. For example, a single nucleotide deletion in *torA*, encoding the TMAO reductase catalytic subunit, renders the entire *torCAD* operon non-functional in a representative *S. baltica* clade E strain, an ecotype occupying preponderantly relatively oxic waters in the redox transition zone of the Baltic Sea where respiration of *N*-oxides is assumed to be less relevant for fitness or survival

Key figure

Bacterial respiration and overview of mechanisms mediating respiration-induced biofilm formation (RIBF)

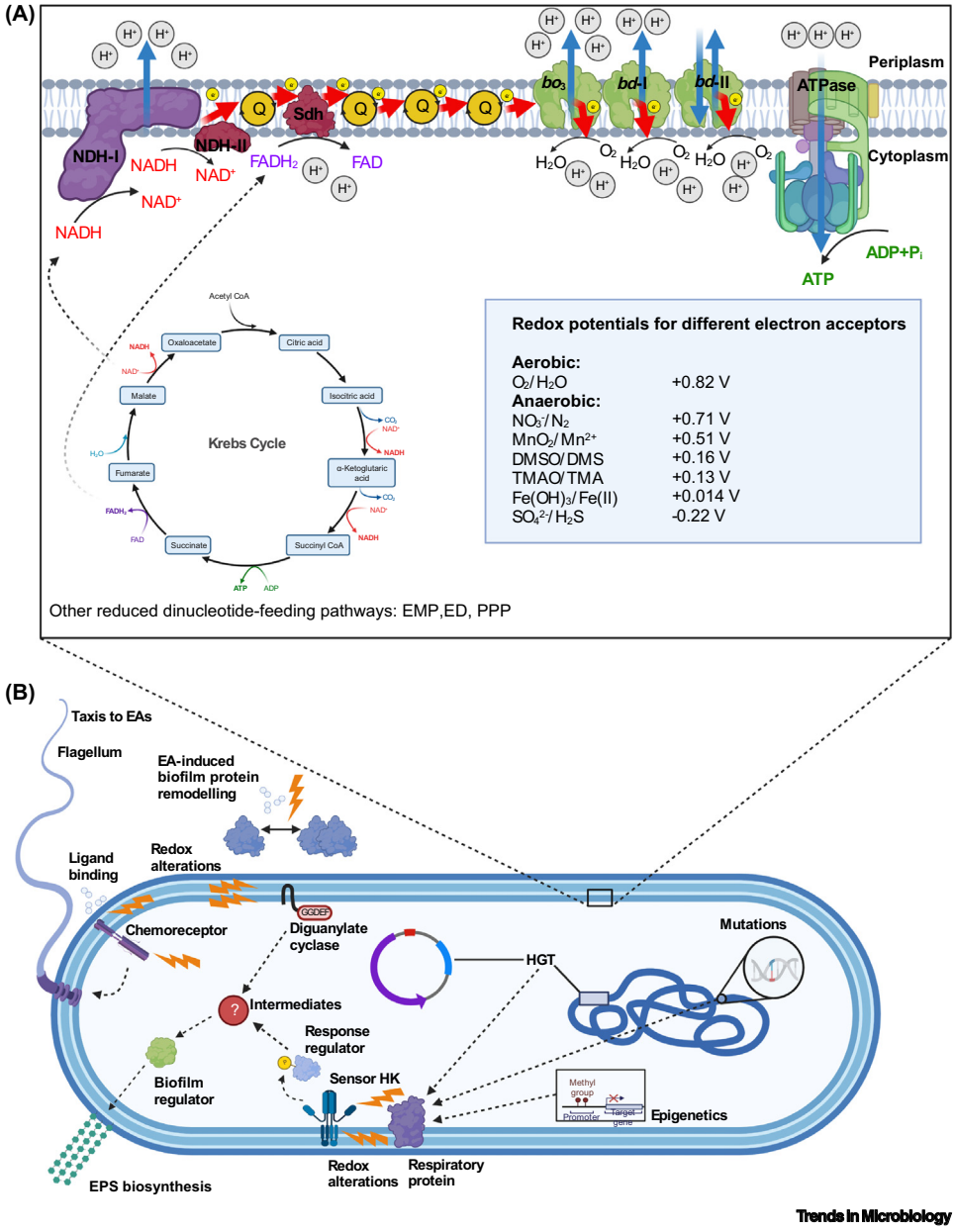

Figure 2. (A) Simplified schematics of the aerobic respiration chain of *Escherichia coli* as a representative model. Reduced dinucleotides from the Krebs cycle or other glycolytic pathways such as the Embden–Meyerhof–Parnas pathway (EMP), the Entner–Doudoroff pathway (ED), or the pentose phosphate pathway (PPP) enter the oxidative phosphorylation chain via type I or II NADH dehydrogenases (NDH-I and NDH-II, respectively), or succinate dehydrogenase (Sdh). Contrary to NDH-II or Sdh, NDH-I couples NADH oxidation with the translocation of four protons to the periplasm, building up a proton gradient. A pool

(Figure legend continued at the bottom of the next page.)

[16]. Loss of the *dmsEFABGH* operon encoding the DMSO reductase complex in a *S. algae* strain associated with a lack of biofilm induction upon DMSO supplementation has been shown [13].

Respiration-mediated biofilm responses are driven not only by intrinsic pathways but may also be subjected to horizontal dissemination [54]. The acquisition of a chlorate respiration composite transposon by *S. algae* strain ACDC might open up for novel regulatory pathways for biofilm formation [55]. An insightful case is that of the human commensal *E. coli* strain Fec10, a recently acquired *E. coli* K-12 clone member. Unlike *Salmonella*, *E. coli* lacks a tetrathionate reductase and is therefore unable to respire on tetrathionate. Acquisition of a tetrathionate reductase from a P1 phage-derived IncY plasmid allows tetrathionate reduction in Fec10, which modulates EPSs biosynthesis in the presence of tetrathionate at physiological temperature [56]. This capacity might contribute to Fec10 colonization of the human gut by triggering a lifestyle switch to take advantage of an electron acceptor that is innately inaccessible to *E. coli*.

Epigenetics

Until now, epigenetic modifications have not been directly linked to RIBF. Nonetheless, a recent study demonstrated the N^6-adenine DNA-methyltransferase ModA2 of *Haemophilus influenzae* to experience phase variation, with the ON switch promoting biofilm formation and downregulating anaerobic respiration pathways, including fumarate and DMSO reduction [57]. This indirectly points to a potential participation of the DNA methylation status in respiration-mediated biofilm turnover. It is expected that a broader implementation of long-read sequencing and base modification analyses will lay the ground for direct functional associations of epigenetic modifications and RIBF in the years to come.

Gene expression

Gene expression variations are key to niche partitioning and ecotype distribution in natural ecosystems [58,59]. It is not yet fully understood whether differences in gene expression could precede genome sequence evolution in response to niche adaptation. Differential expression of clade-specific genes in the presence of environmentally relevant electron acceptors concomitant with transcriptional divergence of shared genes has been implicated in niche specialization by *S. baltica* strains representing distinct ecotypes [19,60]. Comparative transcriptomic analyses

of quinone molecules (ubiquinone during aerobic respiration, and menaquinone/demethylmenaquinone under microaerophilic or anaerobic conditions, collectively represented by 'Q') act as mobile electron carriers at the inner membrane and are key drivers for the generation of the so-called 'electron transport chain'. Electrons finally reach the oxygen reductases (cytochrome oxidases) bo_3, *bd*-I, and *bd*-II, with cytochromes bo_3 and *bd*-I coupling oxygen reduction with a net translocation of protons to the periplasm. The proton gradient is used by membrane-bound ATP synthases for the biosynthesis of ATP. Bacterial respiratory chains are typically branched, containing multiple dehydrogenases and terminal reductases. Omitted components of the *E. coli* aerobic respiration chain include the non-electrogenic NADH:quinone oxidoreductases WrbA, YhdH, and QOR. While oxygen is the most favorable electron acceptor (EA) energetically, diverse alternative electron acceptors (AEAs) can be employed by facultative or anaerobic bacteria. The redox potentials of oxygen and a selection of representative AEAs are presented. (B) Overview of representative determinants and pathways mediating RIBF. These include distinct, horizontally acquired genetic content (HGT, horizontal gene transfer), DNA mutations, or epigenetic factors affecting the expression or function of respiratory proteins. Alterations in the redox state of the periplasm, inner membrane, or cytosol, are detected by sensory modules of an array of signal transduction pathways including N-terminal sensory domains of c-di-GMP-metabolizing proteins, or histidine kinases (HKs) of two-component systems participating in biofilm production. Recognition of alterations in redox homeostasis or EAs by chemoreceptors alter motility and spatial migration of motile strains to direct them towards a suitable niche for a lifestyle switch. Presence and levels of certain EAs can induce post-translational modifications in redox molecular switches (e.g., Cys residues) of cellular or extracellular proteins, thereby introducing reversible modifications that alter protein function for bacterial adaptation to specific (micro)environments. Additional abbreviations shown in the figure are DMS, dimethyl sulfide; TMA, trimethylamine.

of *E. coli* isolates representing commensal, environmental, and laboratory-adapted strains revealed that, under the same culture conditions, the most dysregulated genes were those that are most dissimilar across isolates [61]. This suggests that gene expression regulation could be directly linked to gene sequence diversity.

In response to nitrate supplementation, downregulation of biofilm EPSs production was preponderant among biofilm-producing UPEC strains [22]. UPEC has been shown to upregulate nitrate respiration *in vivo* during human urinary tract infection [62]. Nitrate can be abundant in urine, and either nitrate or nitrate reduction by-products could represent a cue for the promotion of biofilm dispersal (i.e., a planktonic lifestyle) to aid the dissemination of the pathogen.

Sensing and signal transduction

Environmental sensing and signal transduction are known to be intimately intertwined, but the interplay between these processes and respiration is less obvious. Tactic responses include **chemotaxis** and **energy taxis**. In *Shewanella* spp., taxis towards electron acceptors is likely driven by energy taxis and consistently, it has been shown to depend on the metabolic utilization of the effector, that is, respiration [63,64]. This represents a potential direct link between energy taxis and RIBF. Extracellular respiration pathways of soluble (e.g., DMSO) or insoluble electron acceptors (e.g., metal oxides) are common in *Shewanella* spp. Sensing of insoluble electron acceptors poses the intrinsic challenge of the lack of the corresponding effector gradient that is necessary to trigger taxis. Two chemoreceptors with Cache and PAS sensory domains, respectively, in combination with extracellular electron transfer, are responsible for sensing and congregation of *S. oneidensis* MR-1 cells around insoluble metal oxides [65]. These chemoreceptors are likely to sense the intercellular proton-motive force generated by the cellular population in the vicinity of the metal oxide, thereby increasing the swimming speed and the frequency of flagellar reversals in the proximity of the insoluble electron acceptor, to eventually switch to a sessile lifestyle on the mineral surface [65]. In this process, secreted flavins have been recently shown to aid taxis [66]. Energy taxis towards electron acceptors is also used by pathogens like *S.* Typhimurium during colitis. The Tsr and Aer receptors mediating energy taxis to nitrate and tetrathionate, respectively, sense changes in the proton-motif force and redox status of the cell to direct cellular migration towards the colonic epithelium and boost planktonic population expansion through the respiration of these inflammation-induced electron acceptors [67].

Taxis towards electron acceptors is not necessarily mediated by energy taxis. Chemotaxis to nitrate has been recently demonstrated for *P. aeruginosa* PAO1, an opportunistic human pathogen involved in cystic fibrosis, consisting in binding of this effector molecule to the periplasmic PilJ domain of the McpN chemoreceptor [68]. Chemotaxis requires effector concentrations in the micromolar range, contrary to energy taxis that often requires higher effector concentrations. Consistently, chemotaxis towards nitrate was not observed in representative strains of other *Pseudomonas* species like *Pseudomonas putida* or *Pseudomonas fluorescens*, whose native ecosystem is the rhizosphere where nitrate concentrations are assumed to be more abundant than in the infected lung epithelium, supporting a role for nitrate chemotaxis in *P. aeruginosa* pathogenesis [68]. Previously, nitrate sensing and respiration had been shown to modulate biofilm formation in PAO1 [69]. Based on this evidence, it is tempting to hypothesize a role for nitrate chemotaxis and respiration in *P. aeruginosa* colonization of the human lungs during cystic fibrosis. Coexistence of chemotaxis and energy taxis to the same effector is also possible [70].

The tight association between environmental sensing and niche colonization implies intimate links between chemosensing and intracellular signal transduction pathways regulating biofilm turnover, which are increasingly recognized, but yet poorly understood [71]. **Cyclic di-GMP**

(c-di-GMP) is an almost universal second messenger regulating biofilm turnover in bacteria, together with other linear or cyclic (di)nucleotides [72,73]. Aerobic metabolism modulates intracellular c-di-GMP levels in the plant pathogen *Dickeya dadantii* by upregulating the production of a **diguanylate cyclase** and downregulating the expression of a **c-di-GMP**-specific **phosphodiesterase** [74]. Proteins involved in c-di-GMP turnover frequently contain sensory domains located at their N terminus. PAS domains, for example, which are universal in all kingdoms of life and bind small molecules and cofactors, are renowned cytoplasmic redox sensors frequently found in c-di-GMP turnover proteins [18,75]. Bacteria indeed employ a broad range of sensor domains [76,77], and new ligand-binding-domain families are regularly discovered [18,78]. Understanding the diversity of sensing domains and relating them to the recognized signals is a key research need. In an analysis of proteins involved in c-di-GMP homeostasis of 42 *S. algae* strains, six novel sensory domains have been recently described [18], including a novel CSS domain variant in a c-di-GMP phosphodiesterase presumably involved in sensing the redox state of the periplasm [79]. High c-di-GMP content is associated with higher expression of *c*-type cytochromes in *S. oneidensis* MR-1 [80], and represents a primary candidate to mediate RIBF in *Shewanella*. Other nucleotide second-messenger pathways, such as cyclic di-GMP-AMP (cGAMP) signaling, may play regulatory roles in distinct bacterial species. For instance, cGAMP specifically controls Fe(III) particle respiration and regulates a transient surface-associated lifestyle in *Geobacter sulfurreducens,* in contrast to the permanent, c-di-GMP-mediated sessility [81]. This represents an advantage when an insoluble electron acceptor is present as a suspension of small particles, a scenario in which a permanently sessile lifestyle would be counterproductive.

Protein interactions with respiratory components or modifications induced by electron acceptors

In the stratified biofilm microenvironment, *B. subtilis* secretes the biofilm matrix protein BslA, a major contributor to biofilm hydrophobicity, harboring a conserved C-terminal CxC motif. In the aerobic layers of the biofilm, BslA undergoes oligomerization through disulfide bond formation mediated by membrane-bound thiol oxidoreductases and direct oxidation of thiol residues by oxygen [82]. This protects the multicellular community from penetration of pernicious substances into the biofilm while allowing access to nutrients in the oxygen-depleted, hydrophilic layers.

Direct interaction of cytochromes with histidine kinases of two-component systems involved in biofilm production represents a distinct form of RIBF regulation. *B. subtilis* PY79 displays exacerbated colony wrinkling and biofilm production under low oxygen concentration. In *B. subtilis* PY79, the histidine kinase KinB phosphorylates Spo0A, a key determinant of biofilm EPS biosynthesis. KinB forms a complex with cytochromes involved in aerobic respiration. When respiration is impaired and electron transport is therefore low, KinB is activated, presumably with participation of a reduced Cys residue in the second transmembrane segment [30]. Thus, transmembrane segments of histidine kinases, chemoreceptors, or c-di-GMP-metabolizing proteins may recognize the redox status of the membrane and mediate lifestyle-switching responses in function of it. Additional support to this notion is provided by research on *S. aureus*, which also responds with increased biofilm formation to low oxygen levels such as those encountered in certain human tissues like infected wounds. In *S. aureus*, the sensor histidine kinase SsrB responds to the redox status of the quinol pool, phosphorylating the response regulator SsrA under hypoxic conditions, ultimately leading to increased biofilm formation through cell lysis and release of cellular polymers [32]. Interestingly, this response was conserved in four *S. aureus* strains. A similar mechanism operates in *S. aureus* through the SaeRS two-component system regulating fermentative biofilm formation [33].

Applications and future directions

Cells living in a biofilm are generally more tolerant to antibiotic treatment and the action of the host immune defenses. Most chronic infections are caused by bacteria present as biofilms [73]. As RIBF is increasingly recognized, respiratory enzymes mediating biofilm formation, virulence, and fitness *in vivo* may emerge as potential therapeutic targets in diverse groups of pathogenic bacteria [25,83]. Bacterial energy metabolism is already in the spotlight as a target for small molecules aiming to overcome antibiotic resistance of recalcitrant infections by biofilm-forming bacteria [84,85]. Indeed, bacterial respiration and antibiotic efficacy are intimately linked [86]. Bactericidal antibiotics are thought to stimulate cell death by inducing the production of lethal concentrations of reactive oxygen species upon enhanced cellular respiration, resulting in increased production of superoxide radicals that damage Fe-S clusters and lead to the production of even more reactive and pernicious hydroxyl radicals [87]. Conventional antibiotics target metabolically active processes and are therefore largely inefficient against persister cells, which are abundant in mature biofilms and are characterized by exhibiting reduced metabolic activity and ATP biosynthesis. The role of respiration during stationary phase on persister formation by *E. coli* has been demonstrated [88], delineating a target for potential antipersister therapies. Targeting the complex interspecies and interstrain social interactions within biofilms also offers new avenues in the prevention of antibiotic resistance. For instance, the EPSs of *Salmonella* biofilms represents a public good whose chemical inhibition, remarkably, has been shown to select against resistant strains [89].

The strain specificity of RIBF could be exploited in other diverse contexts, such as probiotic engineering. For instance, genome sequence analysis of the vaginal native strain *Lactobacillus iners* AB-1 revealed the presence of a Fe-S cluster assembly system shared mainly by vaginal lactobacilli but infrequent in lactobacilli inhabiting other body niches such as the intestinal tract [90,91]. This points towards a potential involvement of oxidative stress-counteracting mechanisms in adaptation to the vaginal environment. *Lactobacillus* spp. are in most instances non-respiring bacteria, but the presence of *cydABCD* and *men* homologs involved in aerobic respiration is consistent with experimental evidence of increased biomass under aerobic growth in the presence of menaquinone and heme [92–94]. For some species, nitrate respiration through a NarGHJI reductase has been described [95]. It is yet unknown whether respiration affects biofilm formation and/or bacterial fitness of *Lactobacillus* spp. As the effect of probiotics is often limited due to their poor permanence, exploitation of RIBF by proficient strains, or the engineering of RIBF mechanisms, represent novel potential options for the production of probiotics with enhanced biofilm formation and persistence *in vivo* in their respective body niches [96,97].

Biotechnological applications of strain-specific RIBF may naturally extend beyond human health. Rhizobacteria like *B. subtilis* promote plant growth and inhibit the establishment of pathogens by forming biofilms on the plant roots. The root microenvironment is characterized by low oxygen levels and abundance of AEAs such as nitrate, often supplied as fertilizer. Oxygen-limitation-induced biofilm formation has been recently described in the plant-beneficial strain *Bacillus amyloliquefaciens* SQR9 and the underlying molecular mechanisms delineated [98], thereby paving the way towards the screening, selection, and modification of strains of agricultural interest. Proficient respiration-induced biofilm-forming *Shewanella* strains are also amenable to genetic manipulation and employment in microbial fuel cells or bioremediation [99,100], areas that are the focus of significant research efforts with this genus.

Concluding remarks

Collectively, experimental evidence suggests that respiration and lifestyle regulation are intimately linked, beyond the mere need of energy acquisition for the development of cellular processes

Outstanding questions

What are the mechanisms, mediators, and pathways behind RIBF and its strain-specificity?

What is the phylogenetic spread of RIBF and RIBF mechanisms? Are such mechanisms shared by certain phylogroups?

What is the substrate range of RIBF? What are primary substrates for RIBF at a global scale?

What is the ecophysiological significance of RIBF in the context of strain specificity? To what extent does redox specialization and RIBF by distinct strains correlate with occupation of specific environmental niches?

Is RIBF a driver for bacterial speciation?

How, and to what extent, does RIBF affect the composition and spatial structure of mixed biofilms formed by strains with distinct specialization profiles, and multispecies biofilms in a broader context? Are those interactions competitive or cooperative? Do they involve electron transfer?

including biofilm turnover. I argue that regulation of biofilm formation by respiration is widespread and potentially quasi-universal in bacteria. The recently discovered strain specificity of this phenomenon suggests intrinsic specialization by distinct strains, with implications in niche partitioning in diverse stratified ecosystems. As a niche colonization mechanism, I propose that RIBF might drive not only specialization by specific lineages representing distinct ecotypes, but also bacterial speciation itself by directing strains towards new ecosystems. RIBF together with related processes such as extracellular and interspecies electron transfer is likely to enable an array of competitive or cooperative interactions in mixed biofilms by specialized species or strains within the same species. This represents a virtually unexplored aspect of the so-called 'sociomicrobiology'. The ubiquity and strain-specificity of this phenomenon also implies the existence of subtle mechanisms of regulation that we have barely started to discern (see Outstanding questions). Indeed, there is still a substantial knowledge gap on the genetic or, potentially, epigenetic determinants behind RIBF and the different mediators and regulatory pathways involved. A better understanding of these mechanisms across species or strains within the same species will likely provide novel insights into bacterial physiology, and at the same time will lay the ground to applications in multiple areas ranging from biotechnology to biomedicine, including the combat of antimicrobial resistance.

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Declaration of interests

No interests are declared.

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