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Gradients and consequences of heterogeneity in biofilms

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Abstract

Resource gradients influence, and are influenced by, metabolism and our impressions of microbial behavior in diverse ecosystems are not complete without complementary maps of the chemical and physical geographies that influence cellular activities. The interplay between gradient formation and physiology in microbial biofilms has far-reaching consequences for human health, industry, and engineering. In this Review, we discuss examples of gradient formation and physiological differentiation in biofilms growing in diverse settings. We also highlight some of the consequences of physiological heterogeneity in microbial communities, including division-of-labor and increased resistance to stress. A holistic view of microbial multicellularity, incorporating physical and chemical topography as well as the genetically encoded functions that operate within microenvironmental conditions, will be essential for understanding biofilm physiology and its many roles and potential applications.

Introduction

Multicellularity, in which cells adhere to and communicate with each other, is widespread[1] and leads to the formation of physical and chemical gradients, due to physical constraints and endogenous metabolic activity. Such gradients determine the conditions of microenvironments and influence architectural development. Understanding them is key for understanding the physiology of any multicellular structure, making them important areas of study in diverse subfields, ranging from animal development to microbial ecology [2-4]. For example, in the early 1900s, the zoologist Charles Manning Child found that gradients of metabolic activity and O_2 consumption correlated with developmental processes in invertebrate animals and he proposed that metabolic gradients served as the templates for pattern formation in developing embryos [5].

This view was seen as opposing the role of genetics in morphogenesis [6], including genetically encoded signaling gradients, which have now been described for many animals and plants in mechanistic detail [7-10]. More recently, the importance of O_2 gradients in multicellular development has regained recognition and it is now appreciated that an interplay between environmental cues (such as O_2) and genetically encoded mechanisms

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determines morphology [11]. In particular, the implications of O_2 exposure and limitation have taken center stage in processes such as embryogenesis, tumor development, tissue differentiation in plants, and intestinal function (Box 1, Figure 1).

Although leaders in environmental microbiology, such as Winogradsky, Beijerinck, and ZoBell, had already recognized the importance of gradients for microbial communities. in the first half of the 20th century [2-4], it has recently been brought into new focus as an appreciation for multicellularity in the microbial world has become more widespread. Like the cells of animals and plants, microorganisms commonly grow and survive in structures that contain physical and chemical gradients (Figure 2). Microorganisms form multicellular structures when they are confined by the physical environment, when they form intercellular connections, or when they excrete adhesives [1]. Biofilms are a specific type of multicellular structure consisting of microbial cells encased in an adhesive matrix composed of exopolysaccharides, extracellular DNA, and/or proteins [12,13]. The biofilm matrix traps and retains cellular products [14] and supports the accumulation of exogenous materials, which can be modified or degraded by extracellular enzymes [15]. Because it hinders diffusion, the matrix also affects physical and chemical gradients [16-18].

In this Review, and inspired by Child's studies, we discuss the ways in which gradients lead to and result from physiological heterogeneity in multicellular microbial structures (Figure 1b-d), focusing on biofilms. Furthermore, we provide an update on physiological heterogeneity in biofilms (as reviewed previously[19]).

Our original intention for this Review was to describe studies demonstrating mechanistic connections between physical or chemical gradients and in situ physiological effects (such as differential gene expression) across depth in biofilms. However, our literature surveys revealed a cultural divide between approaches that quantify the physical and chemical topographies of biofilms and those that describe physiological mechanisms at the molecular level. Studies that merge these approaches are technically daunting, but are important to do because an understanding of the unique physiology and physiological heterogeneity of cells in biofilms can inform our attempts to control microbial activity in industry, agriculture, and human health. Therefore, we discuss examples that have shown molecular links between gradients and physiology and also examples in which these links are implied.

Gradient formation and division-of-labor

Multicellular structures show spatial heterogeneity both in overall level of metabolic activity and in specific metabolic pathways. In this section, we describe examples of multicellular structures that illustrate the role of gradient formation in promoting physiological heterogeneity, and vice versa. We discuss physiological heterogeneity in the context of metabolism, which is a network of redox reactions that enables cells to obtain energy and produce biomass. Microorganisms show awe-inspiring metabolic diversity, with almost every thermodynamically possible metabolism represented [20]. When subpopulations carry out distinct metabolisms, there is potential for cross-feeding, which can optimize use of resources and thereby maximize growth or survival of the overall group. In this sense, cross-feeding is a form of a division-of-labor: separate subpopulations specialize in

distinct metabolisms and can benefit from their neighbors [21,22]. Although studies of biofilm metabolism tend to focus on one cross-fed metabolite, individual subpopulations are likely to produce multiple distinct metabolites [23,24].

As a context and foundation for understanding these examples, it is also helpful to be aware of a common trend that arises in the chemical topography of microbial ecosystems. This trend stems from the preferential consumption of O_2 over other electron acceptors such as nitrate and sulphate and arises because the reduction of O_2 to H_2O is highly favorable when it is metabolically coupled with most available electron donors [25]. O_2 can also oxidize other chemical species and affect their solubility and bioavailability [20,26]. The degree of oxygenation is therefore usually the primary factor that influences the overall chemistry and microbial physiology of a microenvironment [27]. In many types of biofilms, the source of O_2 is the interface with air or water, or with oxygenic phototrophs near this interface, so O_2 concentrations decrease with depth into the biofilm. Internal microenvironments are more reduced [28,29], enabling the accumulation of molecules such as ferrous iron and sulphide [26].

Escherichia coli*: carbon sources and signals*

Similar to animals (Box 1), microorganisms also show metabolic diversification along O_2 gradients and the potential for cross-feeding, specifically, diffusion of anaerobic metabolites that act as substrates for aerobic cells. For example, *Escherichia coli* can carry out anaerobic mixed-acid fermentation and aerobic respiration [30]. A computer simulation of *E. coli* biofilms growing on glucose predicted metabolic cross-feeding: in the lower, anoxic portion of the biofilm, cells fermented glucose and released acetate, which then diffused upwards and was consumed by cells in the oxic zone. This model was experimentally validated using ^{13}C metabolic flux analysis [31]. In addition, cross-feeding of alanine in *E. coli* biofilms contributed to measurable growth in a specific biofilm subzone [32]. In each of these cases, *E. coli* showed metabolic differentiation that enabled efficient use of carbon depending on microenvironmental conditions.

Metabolites can also act as cues, providing information about the environment and affecting behavior via regulatory responses. When these cues are produced by a cell for the purpose of eliciting a physiological response, they are referred to as ‘signals’. Cue and signal gradients can elicit concentration-dependent responses in microbial communities, similar to morphogen gradients in animals and plants [33,34]. Cues and signals can convey various types of information, including about environmental conditions or the presence and location of other organisms. The extent at which an excreted molecule returns to the producer can be indicative of diffusibility in the surroundings, and such a cue can subsequently stimulate the cell to invest in the production of more costly degradative enzymes that support nutrient uptake [35-37]. For example, *E. coli* and other bacteria release indole, a byproduct of tryptophan metabolism to influence a range of cellular behaviors and properties[38,39]. In the mammalian intestine, indole produced by the microbiota is present in a cross-sectional gradient with high concentrations (up to 1 mM) in the lumen and lower concentrations at the intestinal epithelium. This gradient can control colonization by intestinal pathogens such as *Citrobacter rodentium*, in which indole inhibits virulence gene expression. Indole sensing

may trigger expression of genes involved in epithelial cell attachment in a location where these activities are most likely to promote colonization [40].

Pseudomonas aeruginosa*: exogenous substrates and endogenous metabolites*

Biofilms of the opportunistic pathogen *Pseudomonas aeruginosa* also show the potential for cross-feeding. *P. aeruginosa* is metabolically versatile and able to carry out or catalyze: (i) aerobic respiration, with modular electron transport chain (ETC) components that can be swapped out to tune efficiency [41-43]; (ii) denitrification [44]; (iii) reduction of endogenous compounds called phenazines [29]; and (iv) mixed-acid fermentation [45,46]. *P. aeruginosa* encodes five different terminal oxidases, enzymes that catalyze the final electron transfer step of the aerobic ETC. These terminal oxidases vary with respect to their affinities for O_2 [43], and their expression is differentially regulated [42]. In *P. aeruginosa* biofilms, therefore, respiration not only drives the formation of O_2 gradients through O_2 consumption, but is also itself regulated by O_2 availability [47].

Depending on substrate availability, different subpopulations can use different metabolic pathways and cross-feeding can even enable discrete subpopulations to catalyze subsequent steps in a pathway, such as denitrification [48] (Figure 3a). Phenazine metabolism, which contributes to redox balancing and overall metabolic activity [49], is integrated with respiratory and fermentative metabolisms in distinct ways. Phenazines are redox-active compounds that are reduced by *P. aeruginosa* when other electron acceptors are not locally available and that can transfer electrons to distant oxidants [50,51], such as O_2 in a neighboring biofilm subzone. *P. aeruginosa* produces several phenazines, and the relative amounts of each are influenced by environmental parameters including O_2 availability [47,52,53]. *P. aeruginosa* biofilms therefore contain complex gradients of different types and redox states of phenazines [54]. Microelectrode-based measurement of the redox potential in wild-type (phenazine-producing) biofilms showed that the phenazine pool becomes more reduced with depth, indicating that cells in hypoxic and anoxic zones are reducing these compounds. Mutants lacking specific ETC components form biofilms that are defective in phenazine reduction at depth, implicating the respiratory chain in this activity [29]. Furthermore, *P. aeruginosa* phenazine reduction facilitates redox balancing during mixed-acid fermentation and promotes survival when an oxidant is provided to recycle the phenazines [46]. Moreover, characterization of gene expression in liquid cultures and biofilms suggests that phenazines attenuate denitrification and mixed-acid fermentation, revealing the complex interplay between different anaerobic redox-balancing pathways [48,49].

Regulatory proteins that sense electron acceptors or the availability of other resources mediate metabolic specialization in *P. aeruginosa* biofilms. One such protein is Anr, an ortholog of *E. coli* Fnr. Fnr proteins contain a redox-sensitive Fe-S cluster and have roles in biofilm physiology in diverse contexts [48,55]. The well-studied *E. coli* Fnr is a global mediator of the switch between aerobic and anaerobic metabolism [56-58]. In *P. aeruginosa*, Anr regulates the *cco2* and *nir* operons (involved in microaerobic respiration and denitrification, respectively) so that they are expressed specifically in the hypoxic biofilm subzone [29,48] (Figure 3b-d).

In *P. aeruginosa*, carbon sources are oxidized to provide the reducing power necessary for ATP generation via the ETC. Studies examining gene expression in *P. aeruginosa* in situ suggest links between electron acceptor availability and carbon source utilization. When *P. aeruginosa* biofilms are grown on glucose or pyruvate, hypoxic and oxic regions show expression of an operon that is induced by lactate. This result suggests that cells in the anoxic region (closest to the growth medium) ferment glucose or pyruvate to lactate, which diffuses to hypoxic or oxic subzones and induces gene expression [49,59]. Whether other products of *P. aeruginosa* mixed-acid fermentation (such as acetate and succinate) are also cross-fed in this context has not been examined. The *cox* operon, which codes for a low-O_2-affinity terminal oxidase, is another example of a locus whose expression is affected by carbon source gradients. This operon is expressed in the uppermost portion of *P. aeruginosa* biofilms [47], consistent with its previously reported induction by starvation [42] (Figure 3b-d).

Beyond providing insight into the integration of metabolic pathways, studies of *P. aeruginosa* biofilm energetics have revealed mechanistic relationships between redox state and microbial population-level behavior. When *P. aeruginosa* biofilms are limited for terminal electron acceptors, they increase matrix production, which increases the biofilm surface area-to-volume ratio and therefore O_2 access (Figure 4a). This transition is mediated by RmcA (redox modulator of c-di-GMP), which contains four Per-Arnt-Sim (PAS) domains that function in redox and phenazine sensing [60]. HIF-protein regulatory networks, found in animals, have similar roles in sensing electron acceptor availability and orchestrating multicellular structural changes [61] (Box 1). In both *P. aeruginosa* and *E. coli*, mutations that disrupt ETC function drastically alter overall biofilm organization [29,62].

P. aeruginosa can cause various infections, including life-threatening lung infections in people with cystic fibrosis. Microorganisms in the lung can experience hypoxia due to dense packing and the accumulation of viscous material that limits diffusion [63]. Sputum samples from cystic fibrosis patients contain steep oxyclines [64,65] and vary considerably in redox conditions. The species diversity and potential metabolic versatility of the communities found in cystic fibrosis lungs suggests that metabolite exchange contributes to bacterial growth and survival [66-68]. Indeed, in vitro studies have shown cross-feeding between anaerobic consortia (enriched from the cystic fibrosis lung) and *P. aeruginosa*. Such consortia can degrade mucin, a major carbon source in the lung that cannot be used by *P. aeruginosa*, and convert it into excreted metabolites that support *P. aeruginosa* growth [69].

Cyanobacterial mats: substrate exchange

In plants, O_2-producing tissues are spatially segregated from activities that function optimally under hypoxic conditions, and fixed carbon is cross-fed from photosynthetic tissues to those carrying out non-photosynthetic metabolisms (Box 1). Photosynthetic biofilms, that is, cyanobacterial mats [70], can be several centimeters thick, cover wide areas of a water or sediment surface, and show elaborate gradient formation and physiological heterogeneity. Metabolisms found in cyanobacterial mats include phototrophy and chemotrophy (use of light or chemicals as energy sources, respectively) and autotrophy and heterotrophy (use of CO_2 or organic compounds as carbon sources, respectively).

Cross sections of cyanobacterial mats show layers of differently colored bacteria carrying out distinct metabolisms [71]. Cyanobacteria (blue-green in color carry out oxygenic photosynthesis and fix CO_2; they therefore serve as sources for gradients of O_2 and organic carbon compounds in mats. Other species, such as anoxygenic phototrophs (which are purplish in color and do not produce O_2) and chemotrophic sulphate-reducing bacteria, carry out redox transformations that contribute to gradients of various sulphur-containing molecules. Cyanobacterial CO_2 fixation can alter the pH and availability of dissolved inorganic carbon, which promotes mineralization [72] and stratification of mats. This effect can produce a sedimentary record of biofilm formation and provides important evidence for geologists modeling the chemistry of Earth over time[26,73,74] (Figure 2a).

A fundamental physical feature that influences the chemistry of cyanobacterial mats is the light gradient, which affects the types of photosynthesis and associated redox transformations that can occur at specific depths [75]. Light penetration through microbial mat communities is wavelength-specific, with longer-wavelength light penetrating deeper into mats than lower-wavelength light[76]. Although diverse factors affect the distributions of the many types of phototrophs that can be present in mats, visible layering of bacterial pigments roughly correlates with light gradients (Figure 5). Pigments such as chlorophyll *a*, which have absorption maxima at shorter wavelengths, are present in greater abundance in the upper portions of mats, whereas pigments such as bacteriochlorophyll *a*, which have absorption maxima at longer wavelengths, are more abundant deeper in mats [76-78]. These pigments are found in oxygenic and anoxygenic phototrophs, respectively, and the positioning of anoxygenic phototrophs at depth is also consistent with their use of electron donors produced by anaerobic chemotrophy, such as H_2 and sulphide.

Because filamentous cyanobacteria closer to the top of the mat produce O_2, heterotrophs that can respire this electron acceptor are typically found associated with or just below this layer [79]. Due to the activity of the heterotrophs, the mat becomes gradually hypoxic and anoxic with depth. The O_2 gradient may contribute to stratification of sulphate-reducing bacterial species due to their differing levels of O_2 tolerance [80-82]. Distinct morphotypes of sulphate-reducing bacteria parallel layers of sulphide (produced by sulphate respiration), with overall sulphide concentration increasing with depth [70,83]. In addition to serving as an electron donor for anoxygenic photosynthesis, sulphide can also be used by chemotrophic sulfur-oxidizing bacteria.

Cyanobacteria in the uppermost layers of mats fix CO_2 during the day, then ferment the fixed carbon compounds to produce small organic acids and H_2 at night [73,84]. These products of cyanobacterial fermentation serve as electron donors and/or carbon sources for anaerobic metabolisms, such as fermentation and sulphate reduction, carried out by non-phototrophs. High H_2 concentrations have been measured in mats that contain lower levels of anaerobes (such as intertidal mats, which are subject to fluctuating environmental conditions that disfavor anoxic subzone formation [73]), and in mats that have been treated to inhibit sulphate respiration [84]—consistent with the model that H_2 produced by the cyanobacteria is consumed by non-phototrophic fermentative or sulphate-reducing organisms. Phylogenetic analyses of bacterial populations across depth in submerged mats show that phyla with major representatives capable of fermentative and hydrogen- and

sulphate-dependent metabolisms are abundant in the anoxic zone [85] (Figure 5). Together, these observations reveal that cyanobacterial mats can function as self-sustaining ecosystems in which carbon sources, electron donors, and electron acceptors are made and exchanged.

Non-photosynthetic microbial mats: electron donors and acceptors

Chemotrophic bacteria that can grow in the dark can also form mats and such mats often thrive in harsh environments, including contaminated water and the areas surrounding hydrocarbon seeps on the seafloor [86,87]. They carry out intriguing metabolisms such as those based on sulphur transformations and anaerobic use of methane as an electron donor and carbon source, which can have significant impacts on industrial processes and the global carbon budget [86,88].

Beggiatoa spp., which are filamentous sulphur-oxidizing bacteria, were among the first novel organisms identified by Sergei Winogradsky, who founded the field of microbial ecology [89]. Sulphide can reduce O_2 in an abiotic reaction with a half-life of ~1 hour [90]. *Beggiatoa* spp. grow at the interfaces between reduced and oxic zones, where they obtain energy by coupling the oxidation of sulphide to the reduction of O_2. They have therefore been referred to as ‘gradient-type bacteria’ due to their localization between steep gradients of these substrates [90]. Autotrophic *Beggiatoa* spp. produce fixed carbon that can be used by heterotrophs that associate with the mats [91,92]; they also serve as prey for various microbial eukaryotes[93]. *Beggiatoa* spp. mats have demonstrated the utility of microsensors in defining the chemical topographies of biofilms and are case studies in the exploitation of environmental gradients by bacterial metabolism [90].

Anaerobic use of methane has been observed for microbial mats surrounding gas seeps in the Black Sea, which show layers of calcification and pink and black microbial growth [94]. These mats produce vertical carbonate structures that track gas bubbles rising from the sea floor, and grow radially around the methane source. Sulphate present in the surrounding water acts as the electron acceptor for anaerobic methanotrophic consortia in the mats. In this case, as in *Beggiatoa* spp. mats, electron donors and acceptors are forming opposing gradients. In one exemplary study, labeled methane was used as an electron donor in samples of the mats. High methane consumption occurred in a mat layer that was relatively close to the interface with water and far from the methane source. These results suggest that use of methane as an electron donor relied on a confluence of substrate availabilities and provide another example that microorganisms living in steep resource gradients need to find a ‘sweet spot’ that supports growth [94].

Consequences of physiological heterogeneity

Residence in the physical and chemical gradients of a multicellular structure has consequences that reach beyond the types of energy metabolism that can be carried out by individual populations. In this section we discuss examples of such consequences for diverse microbial communities, many of which have substantial impacts on human activities and health.

Effects on growth and/or overall metabolic activity

Physiological heterogeneity can manifest as quantitative differences in metabolic activity or as differences in growth state. Cells in a specific growth state exhibit properties that tend to correlate—regardless of whether that growth state was brought on by conditions occurring in a homogeneous liquid culture or in a heterogeneous biofilm—because these properties arise from global shifts in gene expression. For example, *Bacillus subtilis* has three distinct cell types that can be distinguished by specific features—production, motility, or sporulation—with each of these correlating with a different overall genetic program. The ratios of these cell types in *B. subtilis* biofilms vary over the course of development [95]. Two-day-old biofilms display all three cell types, with motile and sporulating cells partitioned to opposing ends of the biofilm (at the bottom and top, respectively), and matrix production spanning the middle region of the biofilm. The distributions of these cell types are thought to arise from pathways triggered by microenvironmental conditions. In *B. subtilis* and other microbial species, effects of microenvironmental conditions on matrix production, in particular, can influence the overall morphogenesis of the biofilm [96-98], similar to developmental processes in animals and plants (Box 1) [99,100].

In *E. coli*, the regulatory pathways that control production of matrix and appendages are elaborate, and decades of work have elucidated intracellular signals and mechanisms that determine the physiological status of a cell. These pathways can inform models of the relationship between environmental cues and biofilm stratification [101] (Figure 4b). For example, a study[102] using fluorescent reporters for promoters active in different liquid-culture growth phases (exponential, exponential/stationary phase transition, and stationary) showed complex expression patterns and suggested that growth state was influenced by the opposing gradients of nutrients (from the bottom) and O_2 (from the top) in the biofilms. Generally, exponential phase-like growth occurred in the middle zone, exponential/stationary phase transition-like growth was observed at the bottom, and stationary phase-like physiology was present both at the top and in a discrete band in the bottom half of the biofilm. This physiological stratification correlates with differentiation over depth in respect to matrix composition and cell morphology. Related studies showed that heterogeneous and vertically stratified expression of matrix components is important for the maintenance of biofilm integrity and structure [97,103]. The physiological stratification of these *E. coli* colonies was linked to the development of properties that resemble those found in eukaryotic tissues (Box 1) and that confer resilience, benefitting the overall community[102,104].

Similar observations have been made for *P. aeruginosa*. In one exemplary study, 16S rRNA and rDNA levels were used to quantify ribosomal content per cell from *P. aeruginosa* at various growth stages in homogenous liquid cultures; these values were then applied to biofilm cells to approximate growth state [105]. Cells at the top of biofilms were in exponential-stationary phase transition-like states, whereas cells at the bottom exhibited stationary phase-like ribosomal content. mRNA quantification for biofilm subzones showed that transcriptional activity was highest in the top region. However, this region also contained transcripts coding for the stationary-phase sigma factor RpoS, further indicating that cells there were physiologically similar to those at the exponential-stationary phase transition in liquid cultures. The dormancy of cells at the biofilm base was later

confirmed using GFP-based pulse-chase experiments and correlated with reduced antibiotic susceptibility [106].

Supporting overall metabolic activity is a design goal in the engineering of microbial fuel cells, devices that harvest electricity from cell suspensions or biofilms. Often, these devices carry out dual purposes because they can use different types of waste as feedstock to support biofilm communities that generate current [107], and their utility has been demonstrated in applications such as powering small computers and outdoor lighting [108]. Electrogenicity in pure cultures has been studied extensively in the bacterium *Geobacter sulfurreducens*, which can grow using a poised-potential electrode as its sole electron acceptor. In a recent study [109], *G. sulfurreducens* grew on a poised-potential electrode with labeled acetate as the electron donor. Numerous prior studies had yielded conflicting results regarding the resource gradient that limits growth of these electrogenic biofilms: some had suggested that it was poor access to the electrode for distant cells[110], whereas others indicated that it was poor diffusion of the electron donor through the biofilm[111]. In their system, the authors found that access to the electrode was the main determinant of metabolic activity. In addition to high metabolic activity adjacent to the electrode, they also observed a secondary band of activity at a distance of ~11-12 μm from the electrode, suggesting that cells further from the solid electron acceptor can use a distinct metabolic strategy. Intriguingly, a similar pattern of banding in metabolic activity can be observed in biofilms of *P. aeruginosa* when they are grown under opposing electron donor and acceptor gradients [49].

Effects on the health of hosts

Microbial communities colonize plants and animals and are influenced by the host [112,113], environmental factors [114], and metabolic interactions between microbial subpopulations [115]. Host-associated biofilms can show stratification with a subpopulation at the periphery of the community performing functions that are beneficial to another subpopulation on the inside. In this section, we discuss three examples of this phenomenon, ranging from monospecies biofilms with differentiated subpopulations, to biofilms with several species from the same genus, to diverse communities with hundreds of species from different genera.

During infections by *Yersinia pseudotuberculosis*, which is closely related to the causative agent of plague, host metabolites can trigger differential gene expression in bacterial microcolonies in deep tissue [116]. Experiments using fluorescent reporter *Y. pseudotuberculosis* strains and a mouse model showed that the dense bacterial clusters express *hmp*, which encodes a nitric oxide (NO) dioxygenase, at the periphery of the microcolony (Figure 6a). Host phagocytes surrounding the microcolony produce NO and microcolonies in a host that is defective in NO production do not express *hmp.* When a Δ*hmp* strain is used in the infection model, *hmp* expression is visible throughout the microcolonies, indicating that NO can diffuse to the center when it is not degraded by Hmp. The Δ*hmp* mutant also shows decreased fitness during host infection. Expression of *hmp* at the periphery of wild type colonies therefore serves to protect cells on the inside from NO, and this cooperation supports robust establishment of infection in the host. This example underscores the role of phenotypic heterogeneity in contributing to the success of bacterial communities.

The human oral cavity contains complex microbial communities that show spatial segregation and gradient formation [117,118]. Substrate availability influences biofilm formation and fermentation of sugars from the host diet can promote the formation of steep pH gradients in tooth-associated biofilms [119,120], which leads to the development of caries. Biofilms on teeth that were extracted from young children due to extensive decay were rotund and contained a core population of the cariogenic species *Streptococcus mutans*, surrounded by a layer of non-*mutans* streptococci, followed by an outermost layer of non-streptococcal bacteria[121] (Figure 6b). Co-culturing of *S. mutans* and *S. oralis*, a non-cariogenic commensal, on tooth enamel yielded biofilms with morphology and biogeography similar to those observed on extracted teeth. This structure promoted pH gradient formation and thus dissolution of the enamel (Figure 6c). Furthermore, *S. mutans* benefitted from the spatial segregation of the two species because *S. oralis* protected it during treatment with the antimicrobial chlorhexidine.

The mammalian intestine is a fascinating example of the roles of gradients in host-associated communities due to its steep cross-sectional O_2 gradient (Box 1), which transcends the host tissue to the associated microbiota [113] (Figure 6d). The mucus layer in particular is thought to contain biofilms [122]. The limited amounts of O_2 that are introduced into the intestine are rapidly consumed by colonizing microorganisms. Because carbohydrates and sugars are abundant, they drive (anaerobic) fermentation. Therefore, facultative and aerotolerant microorganisms are situated closer to the epithelium and strict anaerobes are present at the center of the lumen [122,123]. The recent development of an ‘intestine-on-a-chip’, which can be grown with O_2 conditions that mimic in vivo conditions and can support diverse, physiologically relevant microbiota, is an exciting step towards more standardized studies of the gut microbiota[124].

Surviving exposure to antimicrobials

Biofilms can enhance survival during antimicrobial treatment in various ways. Here, we first will define the mechanistic categories relevant for surviving exposure to antimicrobials, then discuss these mechanisms in the context of biofilms and physiological heterogeneity. Mechanisms that contribute to surviving antimicrobials (microbial ‘recalcitrance’) can be categorized as conferring resistance, tolerance, or persistence [125]. Resistance is generally heritable and involves the activity of dedicated proteins that, for example, efflux or degrade the antimicrobial compound. Such proteins therefore allow a population of microbes to grow at antimicrobial concentrations that would otherwise inhibit growth. Tolerance can be heritable or nonheritable but is exhibited by the majority of cells in a population and allows these cells to survive transient exposure to an antimicrobial. Persistence is a nonheritable property exhibited by a minority of cells in a population that survives exposure to an antimicrobial; it can therefore be thought of as tolerance that is restricted to a subpopulation [125].

Cells in biofilms display higher tolerances to antimicrobial compounds and nanoparticles than those in well-mixed liquid cultures [17,126-129]. Because many antibiotics interrupt processes required for cell division, the presence of metabolically dormant populations (for example, persisters) within biofilms can hinder clearance of infections [130-132]. Furthermore,

the biofilm matrix contributes to antimicrobial tolerance [133] by inhibiting diffusion of antimicrobials [17,134] and enzymes embedded in the matrix can also deactivate, modify, or sequester antimicrobial compounds [135]. Some cells in biofilms therefore experience low-level exposures to drugs, which can select for resistance [136]. Finally, biofilm growth favors the acquisition of resistance genes via horizontal gene transfer due to the close proximity of cells and abundance of extracellular DNA [137].

Gradients can accelerate the development of bacterial drug resistance and enhance survival when pathogens are exposed to antimicrobials. A quantitative model showed that the evolutionary pathways that occur in the presence of a concentration gradient of an antibiotic are distinct from those that occur under uniform concentrations [138]. Exposure of *E. coli* cells to a gradient of ciprofloxacin cresulted in rapid evolution of resistance, which did not develop when the antibiotic was provided at a uniform concentration [139]. Incubation of *P. aeruginosa* biofilms with ciprofloxacin had differential effects on metabolic activity over depth, and phenazine production was an additional factor that influenced the profile of metabolic activity in response to ciprofloxacin exposure [49]. *P. aeruginosa* biofilms showed a phenazine-dependent ciprofloxacin tolerance, and enzymes implicated in phenazine reduction were required for this enhanced tolerance, suggesting that the phenazine-supported metabolism that occurs in hypoxic biofilm subzones contributes to survival during exposure to antimicrobials [49]. The fungal pathogen *Aspergillus fumigatus* experiences O_2 gradients during biofilm growth and reduced metabolic activity of O_2-limited cells decreases their susceptibility to antifungals. Provision of O_2 at the biofilm base led to increased metabolic activity and antifungal susceptibility, indicating that increasing O_2 availability could improve treatment of biofilm-based infections [140]. However, many infectious diseases are polymicrobial and different species show differential responses to environmental conditions. Recent work examining the responses of cystic fibrosis sputum microbiota to pH and O_2 gradients indicated that species differentially adapt to distinct microenvironments and that this impacts antimicrobial susceptibility [141]. Accounting for the physiological states and anticipated responses of different species present in an infection could enhance the success of treatment.

Industrial and other applications

Biofilm formation can influence microbial applications, for example, in wastewater treatment, food production, and electricity generation. In many such processes, microorganisms form communities with complex chemical topographies and rely on the interplay of diverse metabolic and signaling pathways. Yet research on these communities tends to focus on the overall process and not the molecular details of gradient formation or physiological heterogeneity. In this section, we provide a brief overview of an example from food production (cheese making) to illustrate the importance of physical and chemical gradients in a microbial application.

The microbial conversion of sugars to acids and/or alcohol is required for production of diverse foods and beverages, including fermented fruits and vegetables, cheeses, teas, and coffee. One of the foundational processes of cheese production is the anaerobic conversion of lactose to lactic acid. Some cheese production methods therefore aim to promote aerobic

processes on the outside, which can produce an edible rind and limit entry of O_2 into the cheese wheel. In cheeses with heterogeneous interiors, such as Emmental and Blue, the production of CO_2 and/or introduction of O_2 gives rise to gas pockets, and the presence of O_2 inside the cheese lead to the growth of mold. In addition to gradients of O_2, salt and pH gradients are also important in the production of cheese [142]. Submersion in brine produces a salt gradient. In Blue cheese, the lower salt concentrations in the cheese interior allow for earlier growth of mold, which results in a faster rise in pH as the mold converts lactic acid to CO_2. Finally, pH gradients can also promote the formation of other chemical gradients; for example, in cheddar production, a pH gradient formed during the salting process can promote formation of a calcium gradient [143]. All of these gradients work in concert to influence the microbial communities and the final characteristics of the cheese (for example, aroma, texture, and taste) [142,144].

Concluding remarks

Molecular mechanisms in biology are typically studied in the context of either signaling or metabolism, with little cross-talk between these subfields. A focus on geneticshas resulted in detailed models of development in organisms across the tree of life (including biofilms [145]), described as the products of signal transduction and regulation of gene expression. However, elements that are still lacking in many models are the fundamental ways in which local chemical and physical parameters influence multicellular behavior. These factors modulate biology by linking signaling and metabolism, and an understanding of the relationships between organisms and their environments requires us to take a holistic view of biological function that includes both sides of the signaling-metabolism dichotomy.

We surveyed the literature with such a holistic view in mind, looking for studies that examined the regulation of metabolism in the context of microbial multicellularity. We found that there was a cultural divide in the literature between descriptions of the chemical and physical gradients in microbial communities and descriptions of cellular physiology in these structures. Observations made in different studies show correlations between gradients and physiological patterns, from which inferences can be made regarding the effects of gradients on metabolism and regulation within microbial communities. However, examining these properties in the same sample and in situ will bring us closer to the goal of showing direct links between microenvironmental conditions and physiology. In this Review, we have highlighted studies providing high-resolution chemical topographies and ones enabling detection of gene expression, overall metabolic activity, and specific metabolisms without disrupting biofilm architecture. It is now possible to combine these approaches and test the roles of specific signaling and metabolic pathways in responding to the unique conditions arising in stratified, densely packed microbial structures. By bridging this cultural divide, we can better understand microbial activity outside the laboratory and may uncover fundamental relationships between microenvironmental conditions and multicellular behavior that are applicable across biology.

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Box 1:

How gradients influence development and metabolism in animals and plants

Embryos

O_2 gradient formation and its effects on metabolism and signaling are crucial for embryogenesis in metazoans [148]. Low-O_2 conditions are required for stages of this process that occur soon after the blastocyst arrives in the uterus. The uterus itself is severely hypoxic and O_2 diffusion into embryonic tissue is further impeded by the development of adjacent fluid-filled cavities. After implantation, the embryo forms the three primary germ layers along an internal O_2 gradient, with conditions befitting the unique processes occurring in each layer. For example, development of the yolk sac requires the relatively high O_2 levels of the endoderm, whereas the production of blood cells and development of blood vessels requires the lower O_2 concentrations of the mesoderm.

Changes in O_2 availability during the process of embryogenesis have consequences for energy metabolism and signaling. Generally, the metabolism carried out in animal cells consists of fermentation, which converts glucose into small organic acids and produces low levels of ATP, followed by oxidative phosphorylation, which oxidizes small organic acids via the tricarboxylic acid cycle and aerobic respiration to fuel most cellular ATP synthesis [149]. As the embryo develops, its metabolism shifts from a reliance on oxidative phosphorylation towards increased use of fermentation. This change is mediated in part by subunits of the HIF family of regulatory proteins, which accumulate under low-O_2 conditions and control the expression of hundreds of genes including major loci coding for metabolic enzymes and others involved in blood vessel formation (angiogenesis) [148]. The metabolic shift then triggers additional downstream effects, because similar to low O_2, fermentative intermediates can also act as signals to control animal development [150].

Tumors

As in healthy cells, subunits of the HIF family play major roles in orchestrating the response to hypoxia in tumor cells [151]. O_2 limitation disrupts oxidative phosphorylation and leads to an increased reliance on fermentation for ATP synthesis, correlating with increased production and excretion of lactate. In tumors, cellular proliferation and the development of the vascular network (which delivers resources and takes away waste products) are dysregulated [152]. This leads to the formation of subzones within tumors, at distances greater than ~100 μm away from the nearest blood vessel, that experience limitation for O_2 and nutrients and the accumulation of metabolic products such as lactate. Hypoxia and variable O_2 availability in tumors contribute to metabolic heterogeneity and recalcitrance to cancer treatments [153]. Interestingly, cellular subpopulations in tumors can engage in metabolic cross-feeding, in which lactate produced by cells in hypoxic regions can diffuse to areas with higher O_2 availability and act as a substrate for aerobic metabolism [154].

Plants

In plants, most O_2 production and CO_2 fixation (that is, photosynthesis) occurs in specialized structures, leading to O_2 gradient formation and the requirement for transport mechanisms that distribute fixed carbon to non-photosynthetic tissues [155]. The localization of O_2 production is important because, similar to animal development, key processes in plant development occur optimally under hypoxic conditions [156]. For example, the meristems (the sites of active growth on plant roots and shoots) exhibit chronic hypoxia and treatments that increase O_2 levels in these regions lead to decreased activity, as indicated by effects on leaf initiation, root development, and various molecular readouts. The ERF-VII family of proteins and the ZPR2 protein are examples of regulators that link O_2 sensitivity to metabolism and development in plants.

Intestine

The mammalian intestine is lined with a polarized epithelium and contains hundreds of microbial species. In the intestine, animal and microbial cells are juxtaposed and gradient formation majorly influences the physiologies of both populations. The intestinal epithelium produces mucus and interacts with the microbiota on its apical side and receives O_2 from systemic blood flow to the endothelium on its basal side. This yields a cross-sectional O_2 gradient that starts with oxygenated blood in the endothelium, continues with consumption of O_2 by the epithelium and by aerotolerant bacteria close to the epithelium, and culminates as O_2 becomes undetectable in the lumen. This gradient is important because it enables O_2 to support the viability of the epithelial cells but enables anaerobic microbial metabolisms to occur in the lumen. Both ingested compounds and microbial fermentation products act as electron donors for consumption of O_2 by host cells in the intestinal epithelium [123]. Increased intraluminal O_2 levels have been identified as a factor that can contribute to dysbiosis and increase susceptibility to infection [157,158].

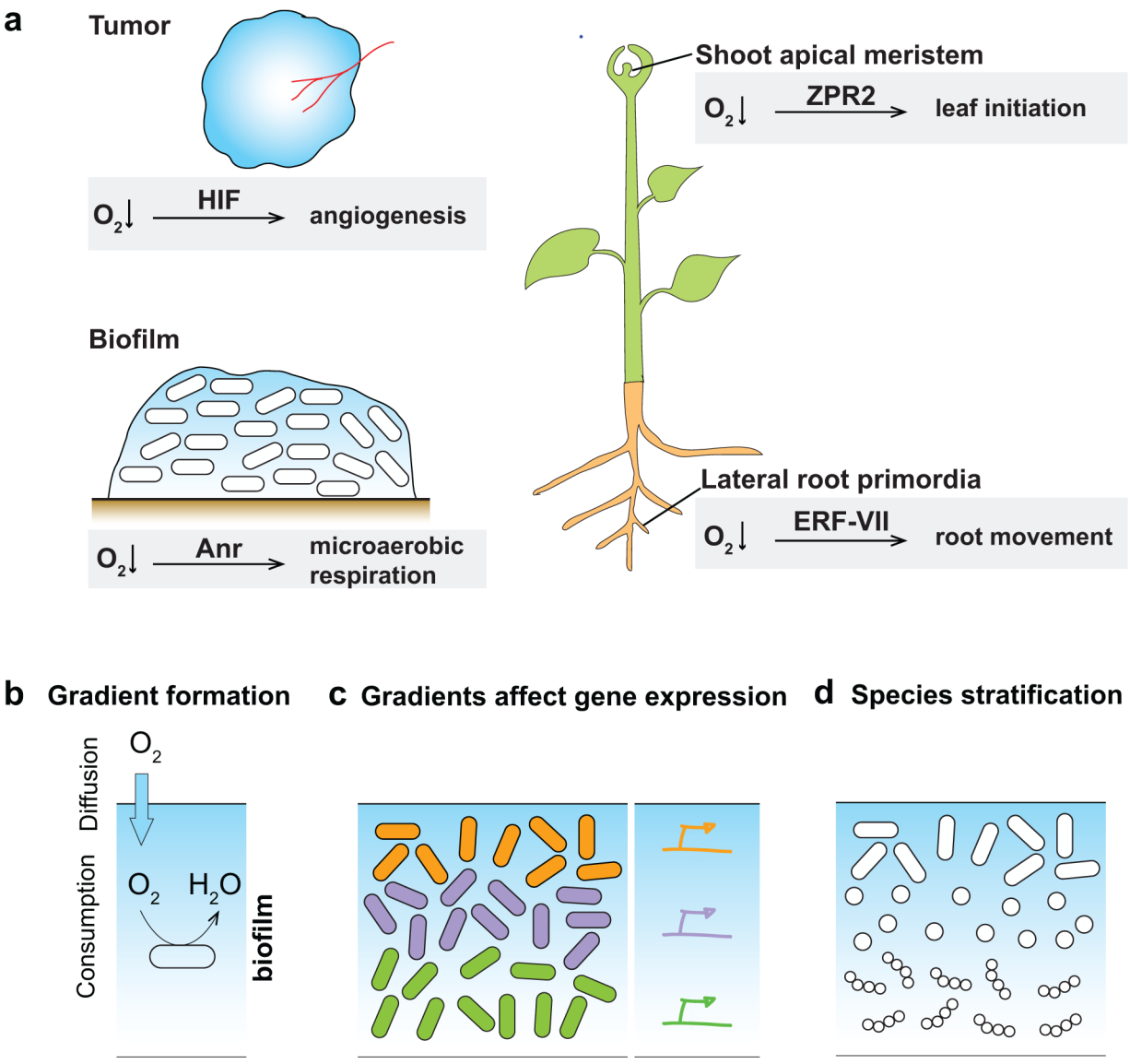

Figure 1. Oxygen gradients influence metabolic differentiation and morphogenesis in multicellular systems.

(a) Schematics of a tumor, a biofilm, and a plant showing regions where O_2 (dark to light shading indicates diminishing oxygen concentration) influences the activity of regulatory cascades that modulate metabolism and/or morphogenesis. Each regulatory protein is part of a pathway that links O_2 sensing to physiological outputs and may play an inductive or inhibitory role. (b) Oxygen (O_2, blue shading) diffuses into a biofilm and subsequent consumption by resident cells leads to O_2 gradient formation. O_2 gradients can result in differential gene expression within a monospecies biofilm (depicted as varying colors; c) and/or stratified growth of species (depicted as varying shapes; d) depending on O_2 preferences. For (b) through (d), the top represents the interface with air or oxygenated liquid.

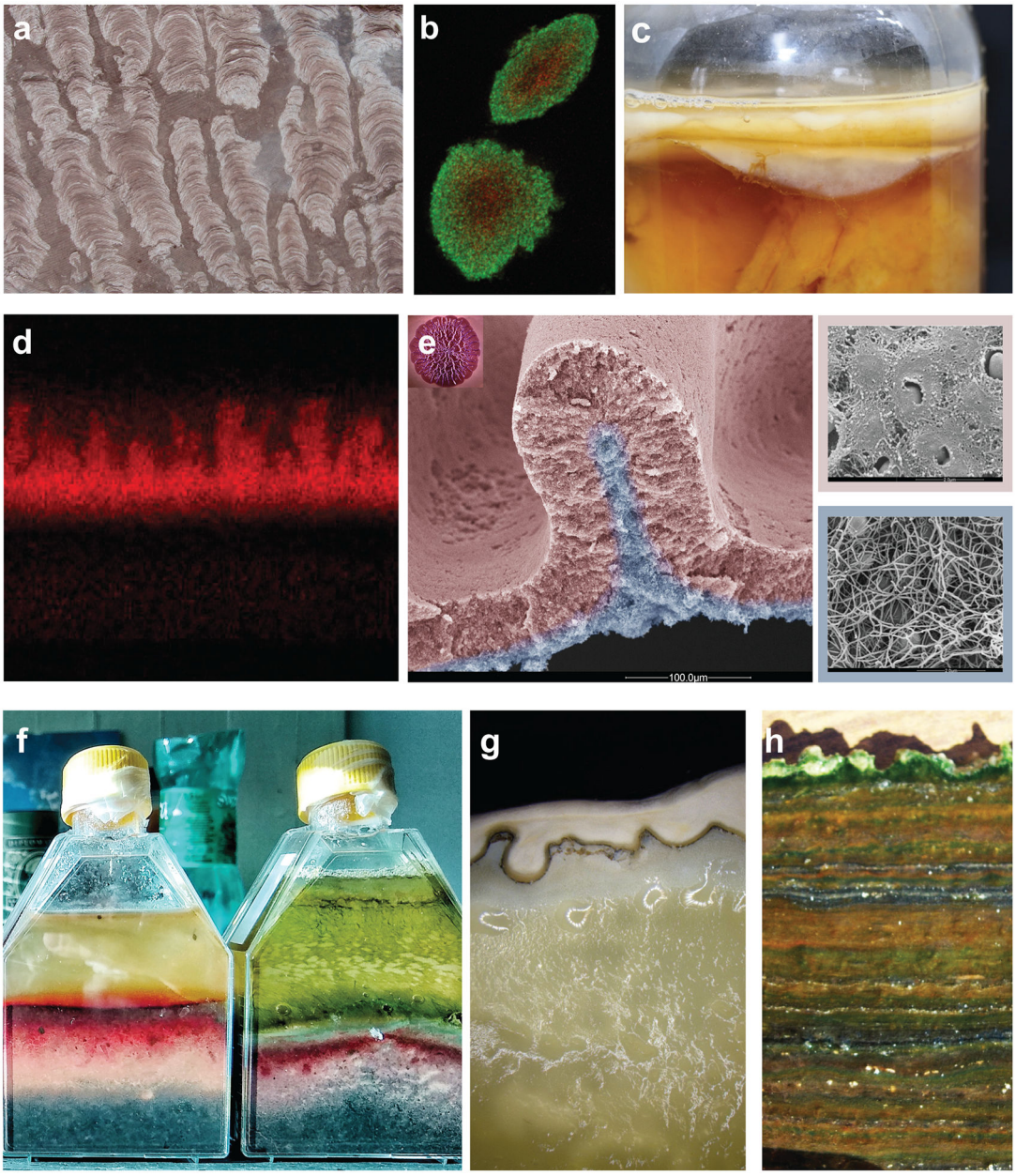

Figure 2. Physiological stratification of biofilms along chemical gradients.

(a) The image shows fossilized stromatolites, which are structures formed by microbial mats that trap sediment and promote mineralization in layers (Satka Formation, Southern Urals; the wider columns are ~10 cm in diameter). (b) When growing in aggregates, *Pseudomonas aeruginosa* shows maximal expression of *narG* (red), which encodes an anaerobically expressed nitrate reductase, in the center (tagging by a universal probe, for rRNA, is shown in green). (c) A layered biofilm, required for production of kombucha, grows at the air-liquid interface of the tea. (d) Cross-section of a *P. aeruginosa* colony biofilm (top: interface with air; bottom: interface with agar-solidified medium) showing discrete zones of metabolic activity (red)[49]. (e) Cross-section of an *E. coli* colony biofilm (left panel, top view of whole biofilm shown in inset), false-colored to indicate discrete architectures that

are visible at high magnification (right panels, top corresponds to the red colored zone in the cross-section, bottom corresponds to the blue zone) (. (f) Stratified growth of bacterial species in Winogradsky columns. These miniature ecosystems develop over several weeks in standing vessels containing aquatic samples that are incubated in sunlight. The enriched species depend on the nutrient conditions of the initial setup. (g) Cross-section of Humboldt Fog goat milk cheese (Cypress Grove Chevre, Inc.) showing the following layers over depth: mold (microbial fungus) at the cheese-air interface, ash (added during production to increase the pH at the surface of the cheese and promote mold growth), runny cheese, and solid core cheese. (h) Cross-section of a cyanobacterial mat from Guerrero Negro, Baja California Sur, Mexico. Part a provided by T Bosak, part b reproduced with permission from ref. [146], part c provided by J. Gowans, part d provided by L. Florek, part e reproduced with permission from ref [97], part f provided by D. Giovanelli, part h provided by J. Spear.

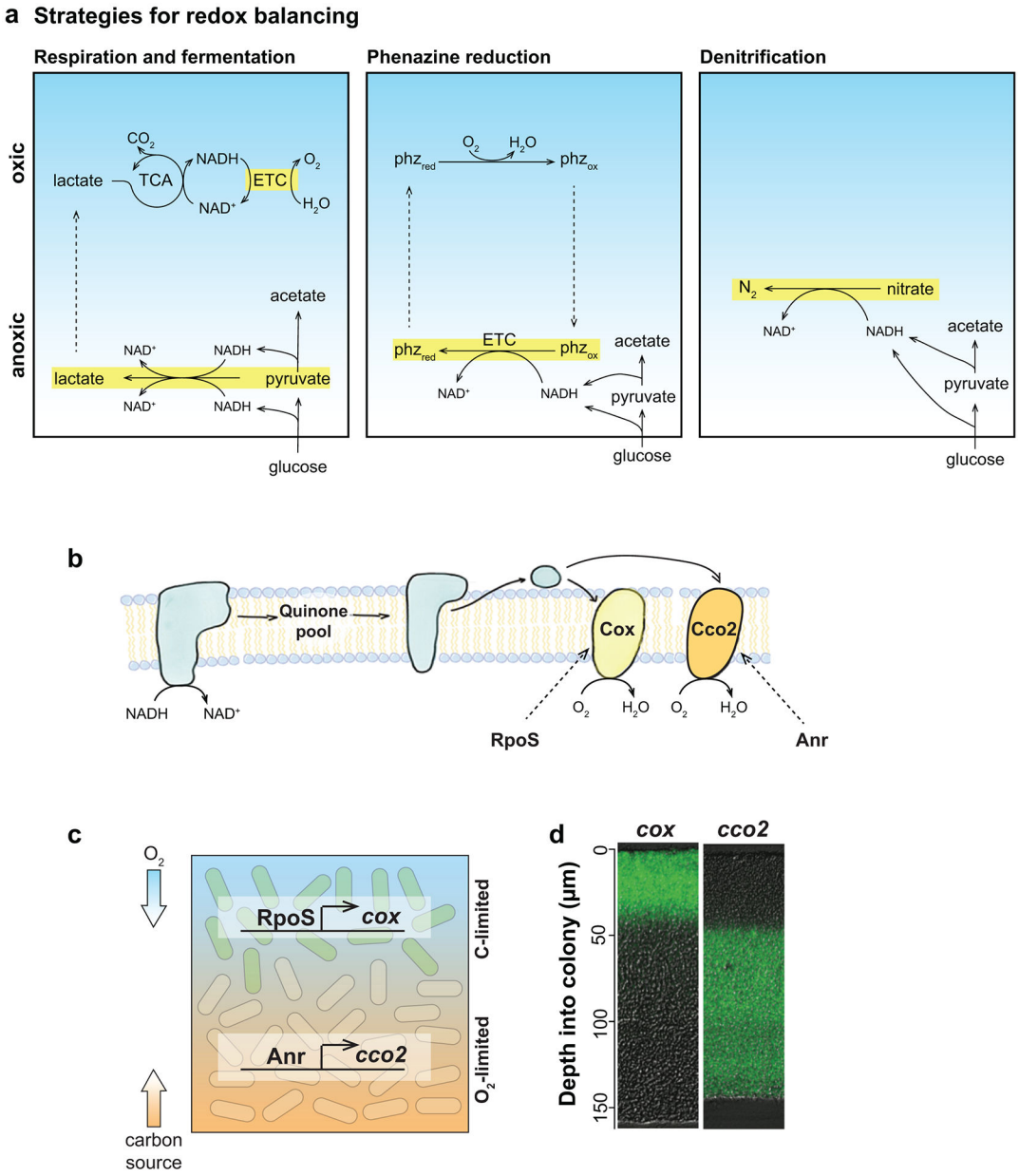

Figure 3. Redox balancing strategies in *Pseudomonas aeruginosa* biofilms.

(a) *P. aeruginosa* can use different metabolic pathways in biofilms. The blue shading represents the O_2 gradient across depth. Yellow shading highlights mechanisms (which are shown in a simplified form) that can be used to reoxidize NADH that is generated through carbon metabolism and the tricarboxylic acid cycle (TCA). The left panel shows aerobic respiration occurring in the upper, oxic zone near the interface with air and mixed-acid fermentation occurring in the lower, anoxic zone at the biofilm base and interface with the growth medium. One of the fermentation products, lactate, can be used as a carbon source and electron donor by cells in the oxic zone. The middle panel depicts mechanisms in which phenazines can contribute to redox balancing. In cells carrying out fermentation, phenazines can reoxidize NADH and potentially promote survival. Mutant analyses have

implicated components of the electron transport chain (ETC) in phenazine reduction in biofilms. Reduced phenazines can be reoxidized in the oxic zone. The right panel depicts denitrification as an additional mechanism through which NADH can be reoxidized. (b) The aerobic ETC of *P. aeruginosa* is modular, such that the final electron transfer to O_2 may be carried out by different terminal oxidases. Electrons from the oxidation of NADH enter the ETC and pass sequentially through the quinone pool and other carriers to the terminal oxidases (yellow), which are encoded by differentially regulated operons. The *cox* operon is regulated by RpoS, whereas the *cco2* operon is regulated by Anr [42]. (c) Opposing gradients of carbon source (orange) and O_2 (blue) lead to microniches within the biofilm. (d) Three-day-old *P. aeruginosa* biofilms show differential expression of respiratory enzymes across depth. The sections shown contain transcriptional reporters in which GFP production is controlled by the *cox* or *cco2* promoters, respectively. *cox* expression is visible in the uppermost portion of the biofilm (left panel), furthest from the carbon source, whereas *cco2* expression is visible in the lower, hypoxic and anoxic biofilm subzones (right panel) [29].

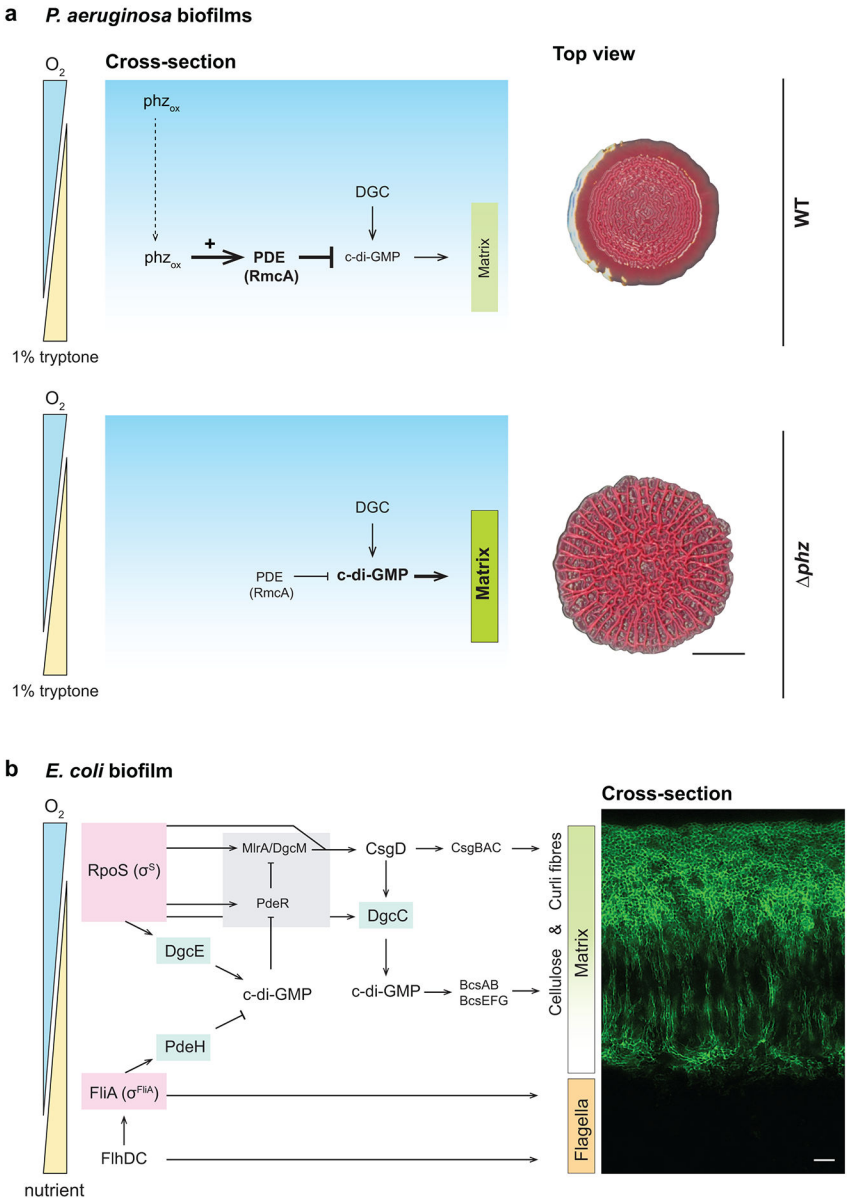

Figure 4. Interplay between resource gradients and biofilm architecture in *Pseudomonas aeruginosa* and *Escherichia coli*.

(a) A regulatory pathway, involving the phosphodiesterase (PDE) RmcA and diguanylate cyclase (DGC), links redox cues (in this case, oxidized phenazines) to matrix production in wild type (WT, top) and phenazine-deficient (Δ*phz*, bottom) *P. aeruginosa* biofilms. Biofilms shown were grown on medium containing 1% tryptone and matrix-staining dyes [44]. (b) Several regulators influence the architecture of *E. coli* colony biofilms. Sigma factors are shaded pink, and activities that modulate levels of cyclic diguanylate (c-di-GMP) and thereby affect matrix production are shaded cyan. The activities of the regulators are influenced by resource gradients and they control pathways with opposing effects on levels of c-di-GMP. This intracellular signal promotes production of cellulose, a component of the biofilm matrix. RpoS also promotes the synthesis of curli fibers, proteinaceous components of the matrix, via c-di-GMP-independent mechanisms. FlhDC is a transcriptional regulator

that controls production of the sigma factor FliA. Both FlhDC and FliA promote production and assembly of flagella, which form a mesh-like structure at the biofilm base (not visible in micrograph on the right). Arrows indicate the overall effects of the indicated regulators and not direct interactions. Additional regulatory steps, some of which have been omitted here for simplicity, are described in detail in [101]. The micrograph at the right shows an *E. coli* biofilm treated with a fluorescent matrix stain, reproduced with permission from [147]. Part a provided by M. Smiley.

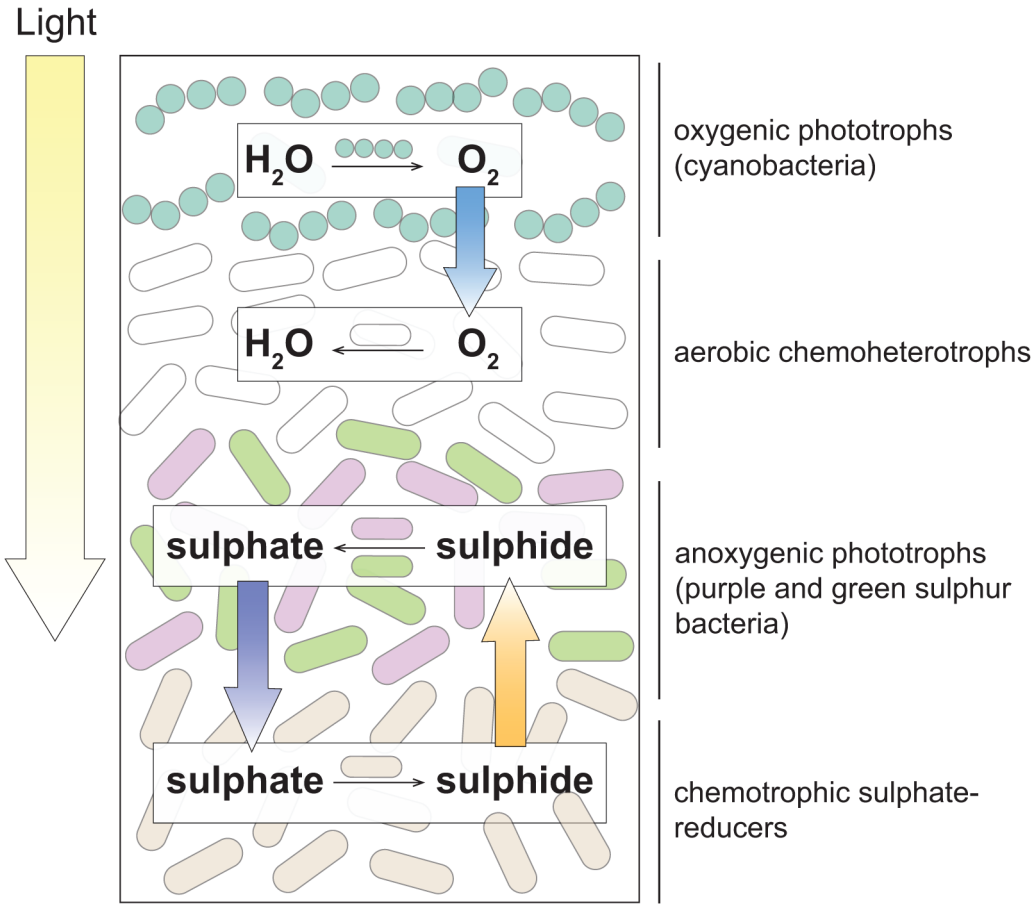

Figure 5. Gradient formation and examples of metabolite exchange in cyanobacterial mats.

This simplified representation shows some of the metabolisms in cyanobacterial mats. Oxygenic and anoxygenic phototrophs produce light-harvesting pigments that are tuned to utilize light at the wavelengths and intensities associated with their specific depth in the mat. Cyanobacteria oxidize H_2O and fix CO_2, producing O_2 and organic carbon that are consumed by aerobic chemoheterotrophs. Purple and green sulphur bacteria oxidize sulphide and produce sulphate, which is transformed back into sulphide by chemotrophic sulphate-reducers.

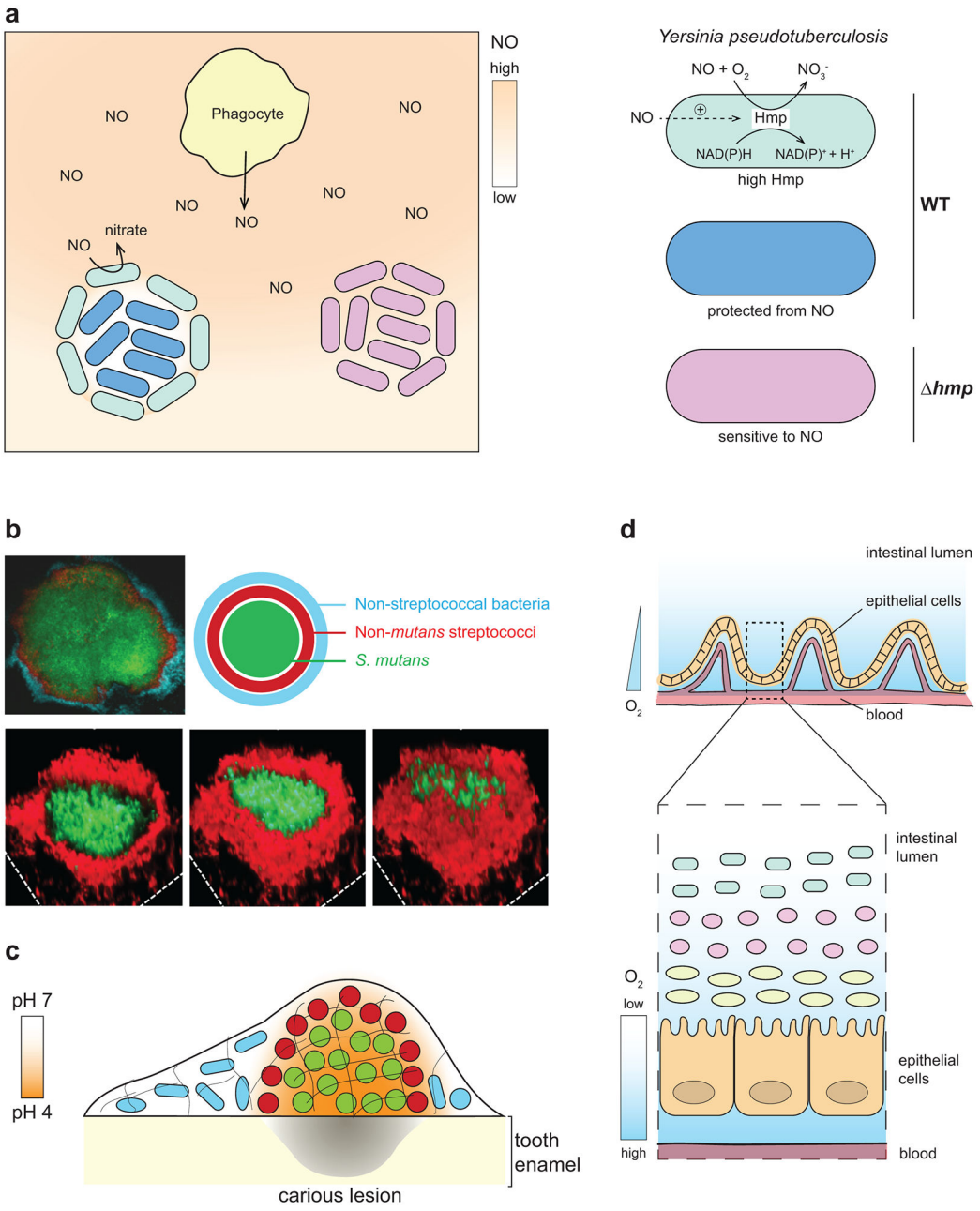

Figure 6. Physiological heterogeneity enhances overall biofilm robustness.

(a) Microcolonies of *Yersinia pseudotuberculosis* grow in the spleen in a mouse model of infection. Host phagocytes produce nitric oxide (NO), and a specialized subpopulation at the periphery of the microcolony expresses the NO-detoxifying enzyme Hmp. NO exposure regulates *hmp* expression with protected cells inside the microcolony are not expressing the gene. Microcolonies formed by a *hmp*-deficient (Δ*hmp*) mutant exhibit *hmp* expression throughout the structure because NO is not degraded. (b) Polymicrobial biofilm from an extracted, diseased tooth exhibit a characteristic rotund structure (top) and uneven distribution of three different categories of bacteria as shown in the model. Bacterial subpopulations were differentiated using fluorescent in situ hybridization and

a fluorescence subtraction method. Co-cultures of cariogenic and commensal species of streptococci form biofilms on enamel in the laboratory, with a rotund structure similar to that observed on teeth. The three panels show a 3D reconstruction of a cluster from a mature biofilm, revealing the segregation of the two species at different heights. (c) Model depicting the cariogenic species (*Streptococcus mutans*; green) localized at the core of a rotund biofilm structure and surrounded by a shell of the commensal species (such as *Streptococcus oralis*; red), which provides protection from antimicrobials. The rotund structure is associated with a low-pH microenvironment that promotes demineralization of the enamel. Non-streptococcal bacteria (blue) are situated outside the shell of commensal streptococci. (d) The schematic depicts the orientation of the endothelium (blood vessel containing oxygenated blood) relative to the epithelial lining the intestine (top). On top of host intestinal epithelial cells, stratified populations of gut microorganisms can be found within the O_2 gradient that originates at the endothelium (blood vessel). Microbial growth in the gradient is influenced by relative levels of O_2 tolerance. Micrographs in part b reproduced from [121].