Review

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Multicellular and unicellular responses of microbial biofilms to stress

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Abstract: Biofilms are a ubiquitous mode of microbial life and display an increased tolerance to different stresses. Inside biofilms, cells may experience both externally applied stresses and internal stresses that emerge as a result of growth in spatially structured communities. In this review, we discuss the spatial scales of different stresses in the context of biofilms, and if cells in biofilms respond to these stresses as a collection of individual cells, or if there are multicellular properties associated with the response. Understanding the organizational level of stress responses in microbial communities can help to clarify multicellular functions of biofilms.

Keywords: adaptation; microbial biofilms; multicellularity; stress response.

Introduction

Bacteria inhabit highly diverse environments ranging from arctic ice to hydrothermal vents and the atmosphere. Thriving in such widely different temperatures, pressures, radiation levels, and chemical milieus illustrates the remarkable potential of bacterial adaptation to abiotic stresses (Gunderson et al. 2016; Rothschild and Mancinelli 2001). Similarly, bacteria can also adapt to and proliferate during biotic stresses caused by, e.g., bacteriophages, protists, or the human immune system (Finlay and McFadden 2006; Gomez and Buckling 2011; Matz et al. 2005; Pernthaler 2005).

The time scales associated with these different abiotic and biotic stresses can vary dramatically. While some habitat conditions, like the temperature at the ocean floor, may remain stable for years, the temperature at the Earth's surface varies regularly with the annual seasons and daytime, and the temperature in environments created by humans may fluctuate irregularly within seconds (Gunderson et al. 2016; Zierenberg et al. 2000). The different time scales of environmental changes are expected to cause different forms of adaptation. Long-term exposure to constant stresses typically results in constitutive changes to the whole cell as observed for extremophiles proliferating in, e.g., high salt, acidic, or extreme temperature conditions. Similarly, very frequent exposures to stress are also expected to result in constitutive behaviors, as observed for numerous phage defense systems (Bernheim and Sorek 2020; Labrie et al. 2010). Such adaptations may be metabolically costly and incur a growth disadvantage if they are deployed constitutively in conditions where a significant part of the growth cycle is without stress. Therefore, stresses that fluctuate on intermediate time scales are expected to cause transient regulatory responses (Poelwijk et al. 2011). Based on experiments in shaking liquid cultures with populations of single cells, our understanding of bacterial adaptation to temporally varying stresses has grown rapidly over the last decades (Storz and Hengge 2011).

Analogous to the different time scales of stress exposure, stresses can also occur on different spatial scales, which is particularly relevant in bacterial communities. Stresses that span over spatial scales that are significantly larger than clonal cluster sizes are unlikely to be recognized as spatially varying stresses by bacteria. However, some stresses occur on spatial scales that are comparable to the bacterial cell size or clonal cluster size, such as

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mechanical shear stress on the exterior of biofilms, and gradients of nutrients within biofilms (Evans et al. 2020; Flemming et al. 2016; Pearce et al. 2019; Serra and Hengge 2014; Stewart and Franklin, 2008). Such spatially-organized stress patterns that arise during biofilm growth are a characteristic feature of biofilms, and may be partly responsible for the emergent properties and benefits that cells obtain within biofilms. However, in contrast to the large body of work on adaptation to temporally varying stresses, little is known about the types of adaptations we expect for spatially varying stresses on the length scale of a few cells.

In addition to the spatially varying stresses that occur inside biofilms due to nutrient consumption and waste product secretion during growth, biofilm communities as a whole may face external abiotic or biotic stresses. As a community, biofilms are highly tolerant to many different stresses, including disinfectants, antibiotics, desiccation, predation, and ecological competition (Hall-Stoodley et al. 2004; Oliveira et al. 2015). Therefore, biofilm formation itself is often considered to be a stress response. The mechanistic origins of the increased stress tolerance have been identified for some cases, yet they often remain mysterious (Bridier et al. 2011; Ciofu and Tolker-Nielsen 2019; Crabbé et al. 2019; Mah and O'Toole 2001; van Acker et al. 2014). To understand the social, cooperative, and competitive nature of biofilms (Nadell et al. 2016), and perhaps as a guide to elusive mechanisms of increased stress tolerance, it can be helpful to consider the different scales that contribute to stress responses in a community. In this review, we describe a categorization of biofilm stress responses according to the spatial scale of the stress and the organizational level of response (Figure 1), highlighting cases in which the biofilm stress response is simply the sum of all unicellular responses, and cases in which there are emergent multicellular levels to the stress response.

Categories of stresses and responses in biofilms

Noting that a key feature of biofilms is their spatial extent, we categorize stresses into those that are spatially localized (Figure 1B, D), and those that globally apply to all cells throughout the community (Figure 1A, C). Global stresses may be spatially uniform, or with a shallow spatial gradient inside the biofilm so that cells in different locations experience different levels of the global stress. In contrast to global stresses, localized stresses are only

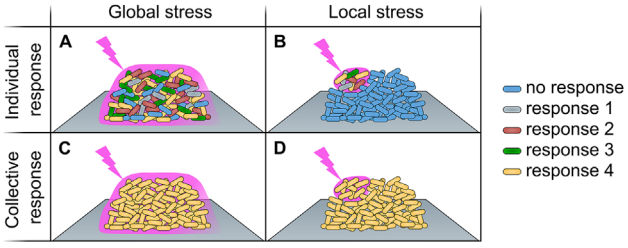

Figure 1: Classifications of the length scales of stresses and the level of response to these stresses in bacterial biofilms.
Stresses may globally apply to all cells in biofilms (panels A and C), or they may be localized to particular regions in biofilms (panels B and D). Responses to stresses can occur at the level of individual cells (A, B) or *via* multicellular processes (C, D). In this schematic classification, blue cells are those that do not respond to the stress, whereas other colors indicate different strengths of stress response; the stress is indicated in pink.

experienced by cells in a particular region of the biofilm. Generally, the response to such stresses could occur at the level of organization of each individual cell (Figure 1A, B), or there could be a collective, multicellular coordination as part of the stress response (Figure 1C, D). These two spatial scales of the stress, and the two organizational levels of the response create four different categories of stresses and responses (Figure 1).

Even though the spatial extent of stresses is often known, placing the responses into the conceptual categories summarized in Figure 1 requires a mechanistic understanding of the process. The discussion of examples of the biofilm stress response in the following sections is therefore limited to cases where the responses have been resolved mechanistically.

Responses of biofilms to global stress

If multicellular features of biofilms, such as those involving extracellular matrix, public goods, cell–cell contact, or cell–cell signaling have no impact on a particular stress response, then the stress response of a biofilm could be the sum of the stress response of each individual cell. In this case, even if a global stress would be applied to the biofilm, the response of each cell could vary inside the biofilm, due to the different phenotypic states of cells in the biofilm, caused by the different cellular microenvironments in the biofilm. According to the classification of Figure 1, this would be an individual-level response to a global stress (Figure 1A). Alternatively, global stresses

could result in changes to the matrix, cell–cell contact, or cell–cell signaling that may be the basis for an orchestrated multicellular response of biofilms to stress (Figure 1C). This section summarizes examples where the organizational level of the response to global stress in biofilms is known.

Global stresses can be applied to biofilms by the addition of a stress-inducing component to the growth medium that can diffuse through the biofilm. For example, many antibiotics can easily diffuse through biofilms, yet highly charged or large antibiotics are prevented from diffusion, unless they are encased in special delivery systems (Diaz-Pascual et al. 2019; Singh et al. 2010; Tseng et al. 2013; Wang et al. 2020). Monitoring all individual cells in *Vibrio cholerae* biofilms during exposure to translational inhibitors revealed that these antibiotics could diffuse throughout the biofilm within minutes. Due to the metabolic consequences of translational inhibition, each cell in the biofilm reacts similarly to cells from liquid culture, by changing their cell size and shape (Diaz-Pascual et al. 2019). In addition to the individual-cell level metabolic response, the translational inhibitors also indirectly caused the degradation of the extracellular matrix, which resulted in a change of the multicellular arrangement in biofilms. These changes of the biofilm architecture rendered *V. cholerae* biofilms susceptible to invasion by other bacterial species or phages. The global stress of translational inhibitors therefore causes responses of *V. cholerae* biofilms at the individual and the collective cell level (Figure 1A, C).

Apart from antibiotics, numerous other molecules cause a global stress in biofilms after adding them to the growth medium, such as nitric oxide, which easily diffuses through the community. The response of *Pseudomonas aeruginosa* biofilms to nitric oxide has been investigated extensively (Figure 2A), revealing that nitric oxide stimulates c-di-GMP-degrading phosphodiesterases, which triggers an intracellular c-di-GMP-mediated switch from the sessile growth state to planktonic growth and biofilm dispersion (Barraud et al. 2006, 2009a). The dispersion response to nitric oxide stimulation is not limited to *P. aeruginosa*, but widespread among biofilm-forming microorganisms (Barraud et al. 2009b). Interestingly, a sudden increase in carbon substrate availability or sudden shifts in the concentration of chemotaxis attractants and repellents also modulate intracellular c-di-GMP levels, to cause dispersal of cells from *P. aeruginosa* biofilms (Basu Roy and Sauer 2014; Morgan et al. 2006; Rumbaugh and Sauer 2020). Not only the addition, but also the removal of a component of the growth medium can cause global stress in biofilms: For *P. aeruginosa*, depletion of the carbon source, iron, or oxygen can trigger an ATP-dependent response that results in the dispersal of individual cells from biofilms, dependent on the intracellular cAMP levels (Figure 2B) (An et al. 2010; Banin et al. 2006; Huynh et al. 2012; Petrova and Sauer 2016). The case studies in which the dispersion mechanisms have been resolved indicate that the dispersal of cells from *P. aeruginosa* biofilms are based on changes in the levels of the second messengers c-di-GMP and cAMP, which are intracellular responses to global stresses (Figure 1A). Changes in c-di-GMP or cAMP prior to dispersion may also lead to the expression of matrix-degrading proteins, which could have an effect beyond the individual cells that produce them, constituting a multicellular element to the response.

In contrast to the individual cell level regulation of biofilm dispersion in *P. aeruginosa*, the initiation of active biofilm dispersion of *Staphylococcus aureus* is based on a collective cell level response to a global signal (Figure 1C), *via* the *agr* quorum sensing system, which controls the production of extracellular proteases that degrade the extracellular matrix (Lister and Horswill 2014). Exogenous addition of the appropriate autoinducing peptide to *S. aureus* biofilm communities also triggers active biofilm dispersal through *agr* activation as a collective response (Boles and Horswill 2008). Similar to *S. aureus*, the active dispersion of *V. cholerae* biofilms requires quorum sensing *via* the master regulator for high cell density HapR (Singh et al. 2017), but in addition, an individual cell level stress response is also required (Figure 2C): Cells must be primed for dispersal by switching to the high-autoinducer state before dispersal can be triggered by the induction of the general stress response sigma factor RpoS, e.g., through carbon source starvation, at the individual cell level (Singh et al. 2017). Therefore, *V. cholerae* biofilm dispersion requires the integration of regulatory elements at the individual cell and multicellular levels (Figure 1A, C).

Another global stress in biofilms that is different from the addition or removal of compounds in the growth medium is a change in the environmental temperature. On length scales between tens to thousands of microns, which correspond to typical biofilm sizes, thermal diffusion is fast so that changes in temperature outside of the biofilm will cause changes inside the biofilm within seconds. Bacterial responses to temperature shifts often do not involve multicellular behaviors (Kortmann and Narberhaus 2012; Phadtare 2004), yet quorum sensing can play a role in the increased tolerance to heat shocks (García-Contreras et al. 2015). Consistent with this, a study of the *Salmonella enterica* biofilm response to heat revealed similar resilience for planktonic and biofilm cells (Scher et al. 2005), suggesting that the response primarily occurs at the individual cell level (Figure 1A). However, for *V. cholerae*, it has been shown that a reduction in environmental temperature from 37 to 25 °C, which *V. cholerae* would experience after

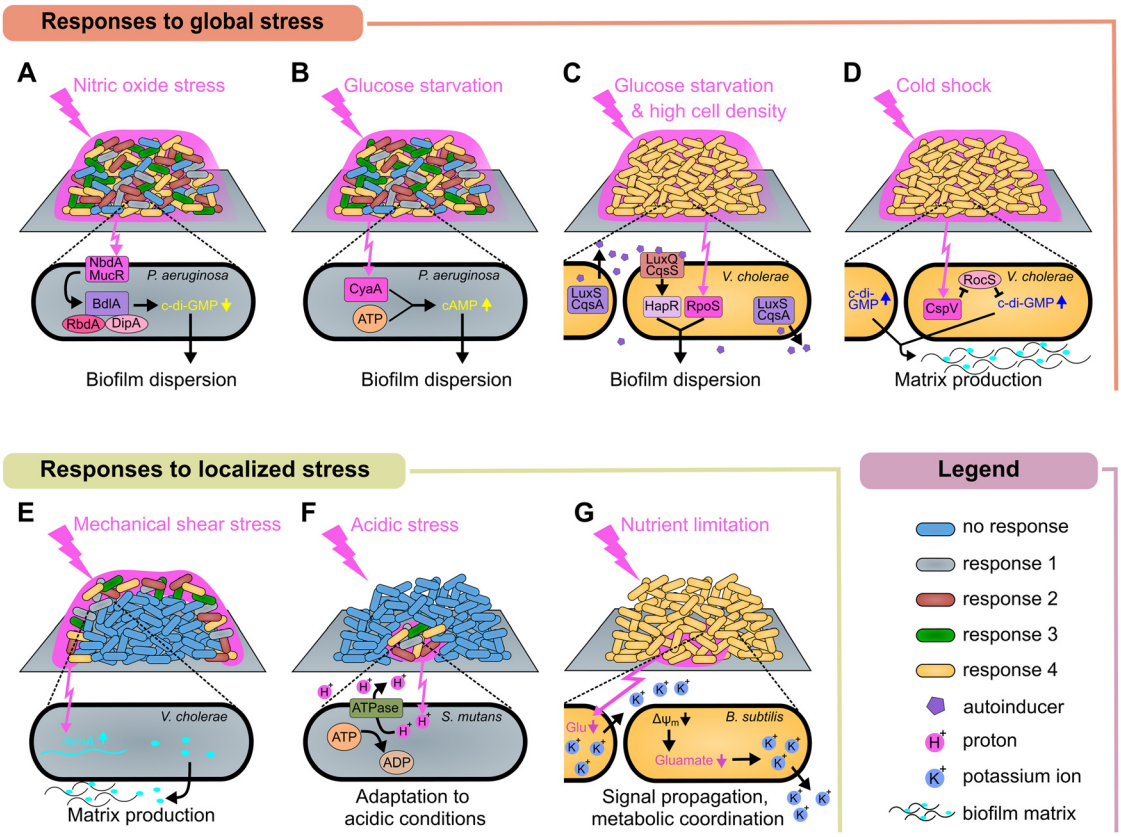

Figure 2: Examples of specific responses to stresses with different spatial patterns.
Responses to global stresses are illustrated in panels A–D, responses to spatially localized stresses are shown in panels E–G. (A) Exposure of *Pseudomonas aeruginosa* biofilms to nitric oxide causes a decrease in intracellular c-di-GMP levels in exposed individual cells, *via* the PDEs MucR, NbdA, DipA, and RbdA with involvement of the methyl-accepting chemotaxis protein homologue BdlA, subsequently resulting in biofilm dispersion (Barraud et al. 2006, 2009a). (B) Removal of glucose from the growth media of *P. aeruginosa* biofilms triggers an increase of intracellular cAMP levels at the single cell level *via* the adenylate cyclase CyaA in an ATP-dependent manner, ultimately resulting in biofilm dispersion (Huynh et al. 2012). (C) Dispersion of *Vibrio cholerae* biofilms after removal of glucose from the growth media requires RpoS, but unlike in panels A and B, dispersion also requires the quorum sensing state associated with a high autoinducer concentration *via* the master regulator for high cell density, HapR (Singh et al. 2017). (D) Cold shock in *V. cholerae* leads to the downregulation of the PDE RocS *via* the cold shock regulator CspV, increasing intracellular levels of c-di-GMP, and subsequent production of extracellular matrix and biofilm formation (Townsley et al. 2016).(E) When *V. cholerae* is exposed to high shear stress, cells experiencing the stress locally upregulate *rbmA* transcription to strengthen the extracellular matrix (Hartmann et al. 2019). (F) *Streptococcus mutans* biofilms display localized acidification inside the biofilm. Several adaptations enable individual *S. mutans* cells to withstand highly acidic pH. One adaptation is based on reverse ATPases that hydrolyze ATP to move protons out of the cell (Baker et al. 2017). (G) Nutrient limitation in *Bacillus subtilis* biofilms causes active release of intracellular potassium, which synchronizes the metabolic activity of neighboring cells *via* a change in membrane potential. Cells receiving this signal in turn release intracellular potassium to propagate the signal throughout the biofilm community (Liu et al. 2015; Prindle et al. 2015).

exiting an infected host, causes elevated intracellular c-di-GMP levels *via* the cold shock regulator CspV and the phosphodiesterase (PDE) RocS (Figure 2D). These higher c-di-GMP levels in turn lead to the increased production of extracellular matrix, which constitutes collective response to this abiotic stress (Figure 1C) (Townsley et al. 2016). Additionally, the temperature-dependent biofilm matrix production in *V. cholerae* is regulated by the temperature-sensitive GTPase BipA, which is highly conserved across different species (del Santos et al. 2020), suggesting that multicellular responses to cold shocks might be common.

Responses of biofilms to spatially localized stress

Stresses that are localized in particular biofilm regions may arise due to biofilm-external factors, such as localized

predation or mechanical shear, or they may arise due to biofilm-internal processes, such as localized acidification or induction of prophages inside biofilms. It is often expected that a localized stress results in a localized response at the level of individual cells. However, there are examples where a localized stress can result in a response that occurs in a larger region than the region where the stress was experienced, involving multicellular behaviors, such as matrix production, public good production, or cell–cell signaling. Mounting a stress response that extends beyond the spatial location of the stress could occur *via* diffusive, advective, or electrical information transfer, yet information transfer within communities represents a relatively high level of community organization and sociality (Nadell et al. 2016). In this section, examples of localized stresses in biofilms are discussed, and whether the response to these stresses occurs at the unicellular level, at the collective cell level, or at both levels.

Mechanical shear stress on biofilms that are grown in the presence of flow primarily affects cells on the flow-exposed surface of the biofilm (Pearce et al. 2019; Stewart 2012). On the inside of the biofilm, where there is typically no flow, the cells could potentially also sense the deformation of the entire biofilm if they could sense the deformation of the matrix (Dufrêne and Persat 2020; Persat et al. 2015). It has frequently been observed that increased fluid flow leads to more compact and mechanically resilient biofilms with different matrix composition (Liu and Tay 2002; Mangalappalli-Illathu et al. 2008), and that fluid shear causes an erosion of the cells in the outer layers of the biofilm, depending on the matrix composition and elasticity (Chambless and Stewart 2007; Stoodley et al. 2001; Telgmann et al. 2004; Xavier et al. 2005). However, it is often not clear if the increased stiffness of biofilms that are exposed to flow results from a localized response of the shear-exposed cells on the outside of the biofilm, or if there is a collective response of the whole biofilm to modify the matrix composition, or if cells that are producing less matrix are simply eroded away from the biofilm surface. Now there is evidence that fluid shear on biofilms causes a localized response in *V. cholerae* biofilms (Figure 2E): It was recently observed that for small biofilms, where the majority of cells are exposed to fluid shear, there was significantly higher average expression of extracellular matrix components that are responsible for cell–cell adhesion compared with low-shear conditions (Hartmann et al. 2019). However, for larger biofilms, where the fraction of cells that are experiencing the mechanical shear is reduced, the difference between the average matrix expression in the low-shear and high-shear conditions is also reduced (Hartmann et al. 2019). A localized response of the cells in the biofilm that are exposed to an increased shear would also be consistent with the recent discovery of a shear rate-regulated operon in *P. aeruginosa* which promises to provide insights into how shear can influence bacterial behavior in biofilms (Rodesney et al. 2017; Sanfilippo et al. 2019). Current evidence therefore indicates that mechanical shear causes a localized response of those cells that are experiencing the stress (Figure 1B), which may include matrix production as a multicellular trait.

Apart from the effects of mechanical shear, flow may also indirectly influence biofilm behaviors by washing away metabolites, autoinducers, or extracellular nutrient chelators, or by transporting nutrients to the biofilm, to influence the behavior of individual cells in biofilms and the collective behaviors of cells in the biofilm (Drescher et al. 2014; Kim et al. 2017; Persat et al. 2015; Singh et al. 2017; Stewart 2012).

The cells on the outer surface of biofilms can also be exposed to predation stress due to protists, phagocytes, other bacterial species, or phages. Cell lysis due to predation can result in a response in neighboring cells that did not directly experience predation. This phenomenon was recently discovered for *P. aeruginosa*, where cells that were lysed by type VI secretion caused a rapid release of antibacterial compounds from neighboring kin cells (LeRoux et al. 2015). Similarly, localized phage infection of *P. aeruginosa* can cause the affected cells to release the Pseudomonas quinolone signaling molecule (PQS), which repels other nearby cells from the infected area, constituting a response in the population that extends beyond the spatial location where the stress occurred (Bru et al. 2019). To predation stresses that are very frequent, such as phage exposure, bacterial species have also adapted by producing particular biofilm matrix components that inhibit phage entry into the biofilms. In *Escherichia coli* biofilms, curli amyloid fibers are a phage-binding matrix component that is produced primarily in the outer regions of the biofilm, where the cells would experience phage exposure (Vidakovic et al. 2018). Such phage-shielding matrix production localized to the outer regions of the biofilm can protect all cells inside the biofilm, even those that do not produce curli fibers. Interestingly, some individual cells inside *E. coli* biofilms also produce curli fibers, which can result in an individual cell level protection from phages (Vidakovic et al. 2018). Phage-shielding matrix components can also be produced everywhere throughout the biofilm, as demonstrated by the matrix polysaccharide in *Pantoea stewartii* biofilms, which provides a size-selective diffusion barrier that inhibits phage entry (Dunsing et al. 2019). Even small reductions of the transport efficiency of phages or other predators into the biofilm are expected to

strongly reduce predation efficiency (Simmons et al. 2018). The matrix components that are known to be involved in predation protection are typically also important for structural and mechanical stability of biofilms and may have even further additional functions (Flemming and Wingender 2010).

Another stress that is localized to cells in the biofilm outer surface is due to antibiotics that are prevented from diffusing into the biofilms, which can be due to particular matrix components that directly bind the antibiotics, or inhibit diffusion *via* size exclusion or electrostatic effects (Ciofu and Tolker-Nielsen 2019; Colvin et al. 2011; Crabbé et al. 2019; Davenport et al. 2014; Keren-Paz et al. 2018; Mah et al. 2003; Tseng et al. 2013). For example, tobramycin, which is a positively charged aminoglycoside, cannot penetrate beyond the outermost cell layers of biofilms of *P. aeruginosa*, causing a localized stress and a response at the individual cell level (Figure 1B) (Tseng et al. 2013).

Analogous to the limited penetration of some antibiotics into the biofilm, biocides can also display a reduced penetration through the biofilm, resulting in a localized exposure of only the cells in the periphery. For triclosan, which is an antimicrobial agent used as disinfectant and antiseptic, the interaction with *S. enterica* and *E. coli* biofilms has been investigated (Tabak et al. 2007; Yazdankhah et al. 2006). These studies have found that the extracellular matrix inhibits triclosan diffusion, and that cells exposed to triclosan respond at the individual cell level by an upregulation of efflux pumps. Further, the exposed cells also upregulate the production of the matrix component cellulose which is a response that provides a benefit for the multicellular community by further reducing triclosan diffusion.

Apart from stresses on the cells located in the outer periphery of biofilms, biofilm-internal processes can also cause stresses that are localized to cells in particular regions of the biofilm. Three-dimensional biofilms display gradients of nutrients and metabolites that are due to the rapid consumption of oxygen and nutrients by the cells that are positioned close to these substrates, compared with the relatively slow diffusion of these substrates into the biofilm. This heterogeneity of microenvironments inside biofilms can result in localized stresses (Flemming et al. 2016; Stewart and Franklin 2008). One example of such biofilm-internal localized stress is the gradient in acidification that occurs as a result of metabolic activity, resulting in highly acidic (pH = 4.3) conditions at the base of the biofilm for dental biofilms (Bowen et al. 2018; von Ohle et al. 2010). In *Streptococcus mutans* biofilms, the cells in the acidic biofilm locations respond individually by extruding protons from the cytoplasm, and by modifying their plasma membrane composition to maintain a more neutral intracellular pH (Figure 2F) (Baker et al. 2017; Fozo and Quivey 2004), constituting an individual cell level response to a localized stress (Figure 1B).

Similar to pH stress in biofilms, oxidant limitation, which results from metabolic activity and the formation of electron acceptor gradients across biofilm depth, is highly localized in biofilms. To mitigate this stress, different forms of extracellular electron transfer are employed, ranging from extracellular cytochromes to nanowires and diffusible redox-active metabolites (Costa et al. 2018; Richter et al. 2009; Wang et al. 2019). For *P. aeruginosa*, the extracellular electron transfer *via* diffusible phenazines has been studied intensively (Glasser et al. 2017; Sporer et al. 2017), revealing that the production of high-potential phenazines is localized to the regions where oxygen is present (Bellin et al. 2014 2016; Recinos et al. 2012), but their reduction is localized to regions that experience oxidant limitation (Jo et al. 2017). Thus, phenazines can provide multicellular benefits beyond the location in which they were produced.

Localized stresses in biofilms that arise as a consequence of community growth can also cause lysis of the cells that experience the stress, which may have benefits for the entire biofilm. For example, regions in the biofilm that have sufficient oxygen, but not enough reductants, may accumulate reactive oxygen species resulting in oxidative stress (Gambino and Cappitelli 2016) and potentially DNA damage, SOS response, and prophage induction. Prophages may also be locally induced *via* small-molecule communication (Erez et al. 2017; Hansen et al. 2019; Silpe and Bassler 2019; Tan et al. 2020). Prophage induced cell lysis was discovered to play an important role in normal biofilm development of *P. aeruginosa*, *E. coli*, *Streptococcus pneumonia*, *Shewanella oneidensis*, *Actinomyces odontolyticus*, because the DNA and cytosolic proteins that are released during cell lysis become a key part of the biofilm matrix (Carrolo et al. 2010; Gödeke et al. 2011; Shen et al. 2018; Wang et al. 2009; Webb et al. 2003). Interestingly, cryptic prophage endolysin induction can also lead to explosive cell lysis, resulting in the release of DNA, proteins, and the formation of membrane vesicles with diverse contents (Turnbull et al. 2016). Independent of prophages, *S. aureus* can also undergo regulated cell lysis during fermentative growth inside biofilms, due to the production of the murein hydrolase AtlA which is regulated by the respiratory regulatory two component system SrrAB (Mashruwala et al. 2017). The products released by the locally lysed cells during biofilm formation, particularly the released DNA, then become important biofilm matrix components for these species (Gloag et al. 2013). Stress-induced localized cell lysis in biofilms therefore

provides benefits to the multicellular community that spatially extends beyond the location of the lysed cells.

As a consequence of substrate consumption of the cells in the biofilm periphery, and diffusion-limited nutrient transport into the biofilm, the cells in the deeper layers of the biofilm are often starved for carbon and nitrogen (Flemming et al. 2016; Lardon et al. 2011; Stewart and Franklin 2008). This heterogeneity in metabolic activity in biofilms is a general feature of biofilms, and cross-feeding is a common process that can maintain metabolic activity in locations below the highly active biofilm periphery (Cole et al. 2015; Dal Co et al. 2019; Wolfsberg et al. 2018). In a series of remarkable recent discoveries for *Bacillus subtilis* biofilms, it has been shown that inner cells of colony biofilms which experience nutrient limitation trigger spatially propagating waves of membrane depolarization (Figure 2G). These electrical signaling waves synchronized the metabolic activity of cells in particular locations of the colony in such a way that cells in the colony periphery periodically arrest growth, which allows substrate diffusion into the colony, resulting in growth even deep inside the colony (Liu et al. 2015; Prindle et al. 2015). This nutrient time sharing for the apparent benefit of the whole community was also observed between neighboring biofilm colonies (Liu et al. 2017), representing an example where a localized stress triggers a multicellular, coordinated response that spatially extends beyond the location of the stress (Figure 1D).

Future challenges and emerging technologies for resolving the organizational level of stress responses

Stresses in microbial biofilms can either be spatially localized or global. Distinguishing whether the cells in biofilms respond to different stresses at the individual cell level, or at the multicellular level, or at both levels together, allows us to clarify how widespread multicellular functions are in biofilms. The classification of the organizational levels of response described in this review requires a mechanistic understanding of the response inside biofilms. A major challenge in biofilm research is therefore the clarification of mechanisms underlying stress responses in biofilms, and whether they differ from analogous stress responses in liquid culture.

In order to distinguish the behavior of single cells from multicellular behaviors, techniques that enable the simultaneous characterization of many single cells are powerful tools. This includes live single-cell microscopy techniques for biofilms (Hartmann et al. 2019), and metabolite imaging techniques (Bellin et al. 2016; Geier et al. 2020). Furthermore, the rapidly improving techniques for bacterial single-cell RNA-seq measurements (Blattman et al. 2020; Imdahl et al. 2020; Kang et al. 2011; Kuchina et al. 2019) are very promising tools for studying the variation between cells, and whether the community behavior is different than the sum of all individual cell behaviors. Single-cell techniques can enable new paths for mechanistic studies of multicellular functions in biofilms.

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