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Minireview

To be or not to be planktonic? Self-inhibition of biofilm development

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Summary

The transition between planktonic growth and biofilm formation represents a tightly regulated developmental shift that has substantial impact on cell fate. Here, we highlight different mechanisms through which bacteria limit their own biofilm development. The mechanisms involved in these self-inhibition processes include: (i) regulation by secreted small molecules, which govern intricate signalling cascades that eventually decrease biofilm development, (ii) extracellular polysaccharides capable of modifying the physicochemical properties of the substratum and (iii) extracellular DNA that masks an adhesive structure. These mechanisms, which rely on substances produced by the bacterium and released into the extracellular milieu, suggest regulation at the communal level. In addition, we provide specific examples of environmental cues (e.g. blue light or glucose level) that trigger a cellular response reducing biofilm development. All together, we describe a diverse array of mechanisms underlying self-inhibition of biofilm development in different bacteria and discuss possible advantages of these processes.

Introduction

Microorganisms are often found in cell clusters, which are held together by an extracellular matrix secreted by the residing cells (Costerton *et al.*, 1995; Hall-Stoodley *et al.*, 2004). Frequently, these microbial communities, denoted biofilms, are attached to a biotic or abiotic substratum (Wahl *et al.*, 2012; Mulcahy *et al.*, 2013; Heindl *et al.*, 2014). Additional forms of such 'sessile biofilms' are microbial mats – laminated biofilms that are comprised of photosynthetic microorganisms in the upper layers, with heterotrophs layered underneath (Decho *et al.*, 2010). In addition to sessile biofilms, microbial films may be formed at air–liquid interfaces, resulting in floating biofilms or 'pellicles' (Beloin *et al.*, 2008; Vlamakis *et al.*, 2013). All these forms of biofilms are the result of developmental processes markedly affecting cell physiology, and thus, cells within a biofilm substantially differ from the planktonic cells from which they originated (Stanley and Lazazzera, 2004; Branda *et al.*, 2005; Lewis, 2008). For example, biofilm cells are highly resilient to a variety of harsh conditions, including antibiotic treatment (Lewis, 2008; Hoiby *et al.*, 2010; Martinez and Rojo, 2011; Lutz *et al.*, 2013; Soto, 2013). Additionally, the close cell–cell association within biofilms may facilitate interspecies exchange of genetic information, and this process of lateral gene transfer may promote evolution (Molin and Tolker-Nielsen, 2003). Nevertheless, planktonic growth may have its advantages under specific conditions. For example, the elevated metabolism characterizing suspended cells compared with cells in a biofilm (Lewis, 2010) may support extensive proliferation of the planktonic cells. Additionally, the high motility characterizing the planktonic cells and the ease of relocation once the inhabited growth niche is exhausted may be advantageous. Therefore, switching between a planktonic and biofilm mode of growth is a critical step in bacterial development, which must be tightly regulated and tuned to environmental cues (Fuqua *et al.*, 2001; Parsek and Greenberg, 2005; Kolter and Greenberg, 2006; Beloin *et al.*, 2008; Karatan and Watnick, 2009; Monds and O'Toole, 2009; Shank and Kolter, 2011; Elias and Banin, 2012; Karatan and Michael, 2013; Vlamakis *et al.*, 2013; Claessen *et al.*, 2014; Fazli *et al.*, 2014).

Inhibition of biofilm formation is a well-known phenomenon. For example, competitive relationships within multispecies biofilms involve the production of non-biocidal substances that diminish biofilm development of other bacterial species and sometimes act between microorganisms as distant as yeast and bacteria

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(Rendueles and Ghigo, 2012). Additionally, numerous mechanisms promoting dispersal of existing biofilms have been revealed; these studies were recently reviewed (Kaplan, 2010; Landini *et al*., 2010; Boles and Horswill, 2011; McDougald *et al*., 2011; Yang *et al*., 2012; Oppenheimer-Shaanan *et al*., 2013; Solano *et al*., 2014) and are not dealt with here. In this review, we focus on mechanisms in which bacteria *a priori* limit their own biofilm development. We describe several examples of such inhibitory processes to highlight diverse mechanisms underlying self-inhibition of biofilm development and discuss their potential advantage.

Biofilm inhibition by self-produced small extracellular molecules

Extracellular secreted small molecules allow intercellular communication between bacteria, which often results in regulation of gene expression in a density dependent fashion, by a mechanism named 'quorum sensing'. These molecules are termed autoinducers in cases where they enhance their own synthesis. Quorum-sensing signal molecules generally promote biofilm formation (Parsek and Greenberg, 2005; Irie and Parsek, 2008; Novick and Geisinger, 2008; Williams and Camara, 2009). However, mitigation of biofilm development by quorum mechanisms is also recognized. Here, we describe several examples depicting biofilm self-inhibition by extracellular small molecules.

Quorum-mediated biofilm inhibition in Vibrio cholerae El Tor. Biofilm formation in *Vibrio cholerae* is reduced at high cell densities (Karatan and Watnick, 2009; Yildiz and Visick, 2009). This bacterium employs two quorum signalling molecules, CAI-1, and an autoinducer found in diverse bacteria, AI-2, which participates in interspecies communication (Ng and Bassler, 2009; Yildiz and Visick, 2009). The culture density is sensed using the principle of a two-component signal transduction system, although in this case, a more intricate mechanism is involved (Fig. 1A). At relatively low cell densities, the kinase domains of CqsS and LuxQ are autophosphorylated. This elicits a multistep phosphotransfer cascade, resulting in phosphorylation of LuxO (Fig. 1A, left panel). The latter, in turn, activates transcription of four small regulatory RNAs (Qrr1-4). These sRNAs, with the aid of the RNA chaperone, Hfq, destabilize the mRNA and prevent translation of HapR, a repressor of genes required for biofilm development (Lenz *et al*., 2004; Bardill *et al*., 2011). Thus, this mechanism de-represses biofilm formation at relatively low cell densities by inhibiting the formation of a biofilm repressor (Fig. 1A, left panel). At high cell densities, however, the autoinducers CAI-1 and AI-2 accumulate and bind to their cognate sensors, CqsS and LuxQ respectively (Fig. 1A, right panel). Under these conditions, the sensor proteins act as phosphatases, subsequently leading to de-phosphorylation of LuxO. The unphosphorylated form does not allow activation of the sRNAs, Qrr1-4. Consequently, *hapR* mRNA is stabilized and translated, and the biofilm repressor is produced at sufficient level (Fig. 1A, right panel). Biofilm inhibition mechanisms employing autoinducers are also present in *Staphylococci* (Novick, 2003; Boles and Horswill, 2011). For example, activation of the accessory gene regulatory system of *Staphylococcus aureus* has an inhibitory effect on biofilm maturation (Boles and Horswill, 2008).

Self-inhibition of biofilm in the cyanobacterium*, Synechococcus elongatus.* Cyanobacterial biofilms inhabit diverse niches and are highly ubiquitous in the environment. Relatively little is known, however, about the molecular mechanisms underlying biofilm formation in these organisms. A recent study of the freshwater unicellular cyanobacterium *Synechococcus elongatus* identified a mutant that forms biofilms under growth conditions that support planktonic growth of the wild-type strain (Schatz *et al*., 2012). The biofilm-forming phenotype is caused by inactivation of a gene encoding a homologue of subunit E of type II secretion systems (T2SE), or PilB of type IV pilus assembly apparati [components of these complexes are homologous, (Filloux, 2004)]. The mutant, T2SEΩ, exhibits aberrant protein secretion. Cell-free extracellular fluid from a wild-type culture (conditioned medium) prevents biofilm formation by the T2SE mutant. This suggests that the planktonic nature of the wild-type strain is a result of a self-inhibition mechanism, which depends on the deposition of a factor to the extracellular milieu (Fig. 1B). Initial characterization of the conditioned medium indicated that the active compound is a heat stable molecule of low molecular weight (< 0.5 kDa).

Currently, only two genes that are essential for biofilm formation were identified in *S. elongatus*. Based on bioinformatic analyses, these genes were suggested to encode a secreted protein characterized by a double-glycine secretion motif and a component of its cognate transport system. Transcript levels of these genes are elevated in the mutant compared with the wild type and are transiently decreased in mutant cells cultured in conditioned medium of wild-type cells (Schatz *et al*., 2012). Thus, it was suggested that an inhibitory compound secreted by wild-type cells governs the expression of genes essential for biofilm development (Fig. 1B). The mechanisms involved in perception and transduction of this inhibitory signal are yet to be elucidated.

The above examples represent biofilm inhibitory mechanisms operating at a relatively high cell density. The signalling molecules in *V. cholerae* are autoinducers typical of the quorum-sensing response in this bacterium

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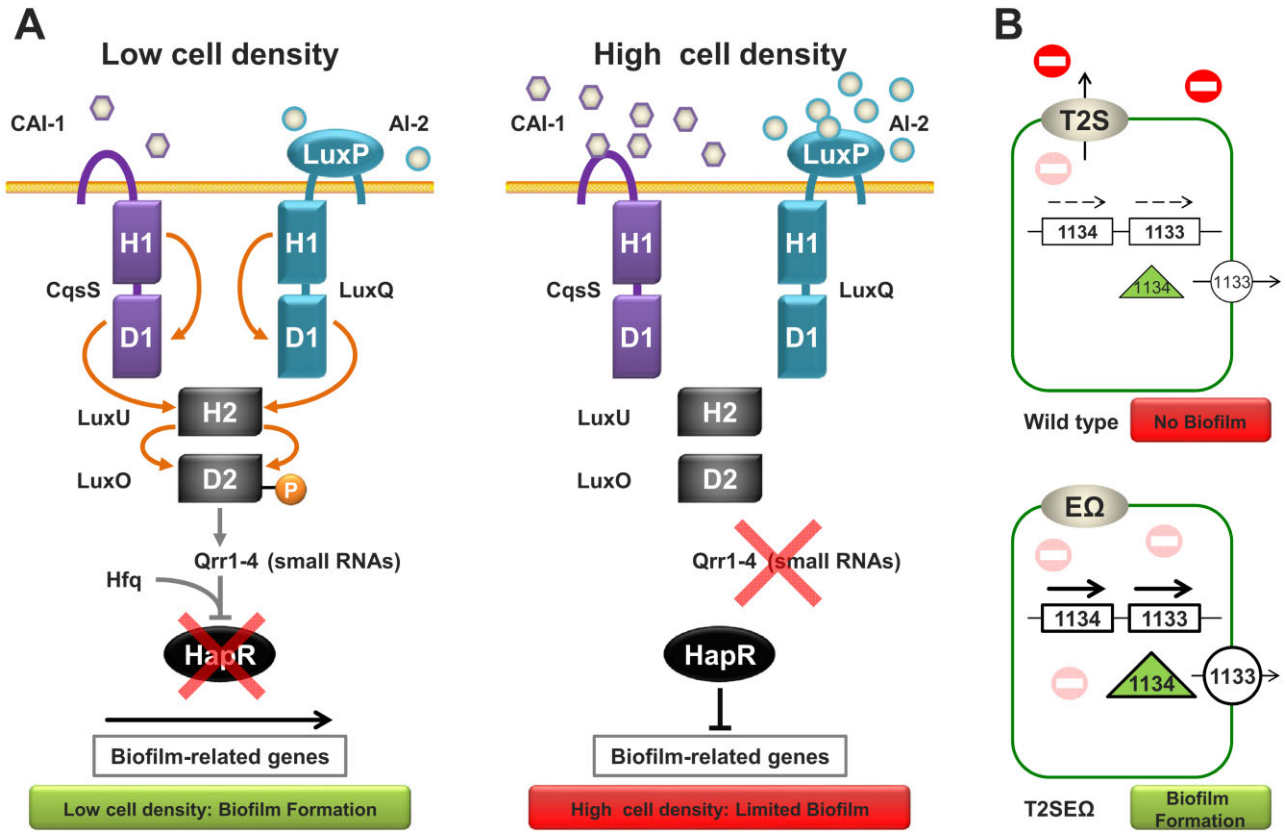


Fig. 1. Biofilm inhibition by self-produced small extracellular molecules.
A. Biofilm inhibition in *V. cholerae* El Tor by quorum sensing. Left panel depicts low cell density and consequently low concentration of the autoinducers, CAI-1 and AI-2. Under these circumstances, the sensor proteins CqsS and LuxQ do not effectively bind the autoinducers; thus, autophosphorylation takes place, initiating phosphotransfer events (orange arrows), ultimately leading to phosphorylation of LuxO. The latter induces expression of the small RNAs Qrr1-4, which together with the chaperone Hfq prevent the production of HapR, a suppresser of biofilm development. At high cell density (right panel), the extracellular concentration of the autoinducers builds up. CAI-1 binds CqsS, while AI-2 modulates LuxQ via binding to the periplasmic protein, LuxP. Under these conditions, the sensor proteins act as phosphatases, leading eventually to dephosphorylation of LuxO. This prevents expression of the small RNAs Qrr1-4; consequently, HapR is produced, and biofilm formation is reduced. H1/H2 represent domains or proteins with histidine kinase/phosphatase function; D1/D2 represent receiver domains or proteins of two component signal transduction systems, which are typically phosphorylated on an aspartate residue. The scheme is modified from Karatan and Watnick (2009) and Yildiz and Visick (2009) and was simplified to present only inhibitory pathways responding to extracellular small molecules.
B. Biofilm inhibition in *S. elongatus* by an extracellular inhibitor. Wild-type cells produce and secrete an inhibitor (red 'no-entrance' sign) that suppresses transcription of the genes Synpcc7942_1133 and Synpcc7942_1134. These genes, which are essential for biofilm formation, encode a putative component of a transport system and a protein characterized by a double-glycine secretion motif respectively. Inactivation of *t2sE*, encoding a homologue of subunit E of type two secretion systems (T2S), enables biofilm development. The biofilm-forming mutant, T2SEΩ, is most likely impaired in secretion of the inhibitory factor, and therefore expresses 'biofilm-genes' at a higher level, and develops biofilms. Dashed and thick arrows represent low and high transcript levels respectively. The T2S system may not be directly involved in secretion of the inhibitor. EΩ indicates impairment of subunit E of the T2S system.

(Ng and Bassler, 2009; Yildiz and Visick, 2009), and the inhibitory signal of *S. elongatus* accumulates in correlation with increasing cell density (Schwarz laboratory, unpublished). Is there an advantage to a biofilm inhibitory mechanism that is activated at a relatively high cell density? It is possible that the inhibitory pathway of *S. elongatus* evolved to prevent aggregation and thereby to maximize light absorption for photosynthesis. Such a 'community escape response' was reported for the photosynthetic bacterium *Rhodobacter sphaeroides* (Puskas *et al.*, 1997). On the other hand, biofilm formation may at times be advantageous; thus, depending on environmental cues, the inhibitor may be downregulated, enabling biofilm development to occur.

In the case of *V. cholerae* or other heterotrophs, suggesting advantages of biofilm limitation at high cell densities is highly speculative because in many bacterial species, biofilm formation is actually enhanced under these conditions (Parsek and Greenberg, 2005; Irie and Parsek, 2008; Novick and Geisinger, 2008; Williams and Camara, 2009). It is possible that under high cell density and consequently exhaustion of the growth niche and

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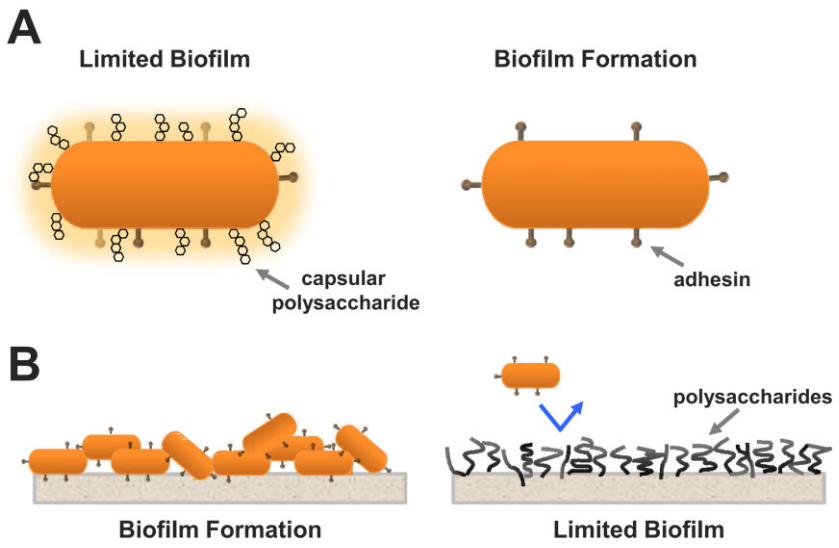


Fig. 2. Biofilm inhibition by polysaccharides.
A. Capsular polysaccharides interfere with adherence. Masking of adhesion molecules limits biofilm development (left drawing). Acapsulated mutants (right) exhibit robust biofilm development compared with the parental strain.
B. Extracellular polysaccharides affect the physicochemical properties of the surface and prevent adherence. Type II capsular polysaccharides and PAM galactan of *E. coli* and *K. kingae*, respectively, act by this mechanism, which results in reduced biofilm formation by the producing bacterium, as well as by diverse microorganisms.

nutrient starvation, those bacteria that are better survivors settle and minimize metabolism until nutrients are replenished, whereas in other species, self-inhibition of the biofilm was adopted to enable cell dispersion.

The role of polysaccharides in biofilm inhibition

Polysaccharides are essential for biofilm development as key components of the biofilm matrix (Branda *et al*., 2005; Flemming and Wingender, 2010). Additionally, a recent study demonstrates that these glue-like substances promote biofilm development by conditioning the substrate surface. In the case of *Pseudomonas aeruginosa* PAO1, the Psl polysaccharide is secreted as the bacteria move on the substrate surface, leaving a trail resulting in further accumulation of bacteria, thus promoting biofilm development (Zhao *et al*., 2013). In contrast to the biofilm-promoting role of polysaccharides, the inhibitory activity of bacterially produced polysaccharides on biofilm formation emerges as a widespread mechanism for limitation of biofilms (Rendueles *et al*., 2013). In many cases, the inhibitory effect is exerted by the presence of the bacterial capsule. In addition, polysaccharides, which are released to the extracellular environment, were demonstrated to decrease biofilm development (Rendueles *et al*., 2013). Below, we briefly describe each of these mechanisms.

Capsular polysaccharides and biofilm inhibition. Studies of diverse bacterial species indicated that impairment of capsular polysaccharide synthesis promotes biofilm development. Namely, wild-type strains generally exhibit limited biofilm formation, whereas the capsule mutants are characterized by robust biofilm development (Joseph and Wright, 2004; Honma *et al*., 2007; Flahaut *et al*., 2008; Kouzuma *et al*., 2010). These studies suggested that capsular polysaccharides mask adhesion molecules that are presented on the cell surface (Fig. 2A), thereby preventing the initial interaction of the bacterium with the substratum, an essential step in biofilm development. Support for a mechanism in which the capsule shields short bacterial adhesins is provided by experiments demonstrating that overexpression of the adhesin Ag43 promotes biofilm formation only in the absence of capsular polysaccharides (Schembri *et al*., 2004).

In summary, because the degree of encapsulation appears to be a critical parameter modulating biofilm development, it is possible that the ability of a bacterium to change the expression of capsular polysaccharides in response to ambient cues may support regulation of biofilm development. Thus, conditions triggering heavy encapsulation will diminish biofilms, while those that do not support encapsulation enable biofilms to form.

Biofilm inhibition by extracellular polysaccharides. Polysaccharides that are released into the extracellular milieu were shown to decrease biofilm development by competing species (Rendueles *et al*., 2013). Here, we describe two cases, in which the extracellular polysaccharides also auto-inhibit biofilm formation by the producing bacterium.

The inhibitory activity of extracellular polysaccharides on biofilm formation was initially revealed by Valle and colleagues, who examined the interaction between uropathogenic *Escherichia coli* (UPEC strain CFT073) and commensal *E. coli*, strain MG1655 (Valle *et al*., 2006). The conditioned medium of the CFT073 strain reduced biofilm development by the MG1655 strain, as well as by a large variety of bacteria, including Gram-negative and Gram-positive species. A screen of random transposon insertion mutants revealed that impairment of

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synthesis of group II capsular polysaccharides abolished the inhibitory effect normally exerted by extracellular fluids. Furthermore, this study demonstrated that the active compound in biofilm inhibition is a high-molecular weight (> 500 kDa) group II capsular polysaccharide that prevents bacterial adhesion and subsequent biofilm development by altering the physicochemical properties of the substratum. The acapsular strain of CFT073, resulting from inactivation of *kpsD*, exhibits robust biofilm formation, which is severely reduced upon application of conditioned medium of the parental strain (Valle *et al*., 2006).

An additional study demonstrated that cell-free extracts prepared from *Kingella kingae* colony biofilms inhibit biofilm development of this strain, as well as biofilm formation by phylogenetically diverse bacteria and by the yeast *Candida albicans* (Bendaoud *et al*., 2011). Preliminary characterization of this extract indicated that it contains abundant DNA, and it was therefore named poly-DNA-containing anti-adhesive material (PAM) extract. Coating of polystyrene surfaces with this material reduces biofilm development. Purification of the PAM extract resulted in identification of a novel polysaccharide, PAM galactan, which limits biofilm formation. Bioinformatics approaches identified a gene cluster, putatively encoding the enzymes required for the synthesis of PAM galactan. Expression of three of these genes, *pamABC*, in *E. coli* resulted in production of PAM galactan. Furthermore, extracts prepared from *E. coli* expressing these genes possess biofilm inhibiting activity (Bendaoud *et al*., 2011).

In summary, studies in both *E. coli* and *K. kingae* demonstrated that bacterially produced exopolysaccharidesare capable of surface modification acting like surfactants and decreasing biofilm development (Fig. 2B). These properties of polysaccharides pave the way for numerous applications aimed at prevention of detrimental biofilms (Rendueles *et al*., 2013).

Self-inhibition by extracellular polysaccharides was demonstrated for *E. coli* and *K. kingae*; however, in many other cases of biofilm inhibition of competitor species by exopolysaccharides, the inhibitory effect on the producing bacterium was not examined (Joseph and Wright, 2004; Davey and Duncan, 2006; Honma *et al*., 2007; Flahaut *et al*., 2008; Kouzuma *et al*., 2010). Given the non-specific inhibitory effect of extracellular polysaccharides, which results from alteration of the surface physicochemical properties, it is conceivable that biofilm limitation by the producing bacterium occurs widely. Namely, self-inhibition alongside broad spectrum biofilm inhibition by extracellular polysaccharides may be a prevalent phenomenon. Is there a selective advantage of such a mechanism? Production of antibiofilm polysaccharides by bacteria that reside in a biofilm may prevent colonization by competitors (Rendueles *et al*., 2013). In cases where the biofilm inhibiting polysaccharides are released at the planktonic stage, we suggest that the producing bacterium induces, at later stages, enzymes that decompose or alter the polysaccharides blocking the substratum. Thus, initially, when planktonic growth is preferred, the exopolysaccharides are produced; subsequently, the obstacle is removed and biofilms develop. This enables inhibition of biofilm development when planktonic growth is advantageous, while 'reserving' the substratum for further biofilm formation. It is also possible that the producing bacterium and its competitors are characterized by different adhesion capabilities. Thus, under particular conditions, e.g. in the presence of moderate concentrations of exopolysaccharides and partial coverage of the substratum, biofilm inhibition does not affect the producing bacterium.

Inhibition of cell adhesion by extracellular DNA (eDNA)

The extracellular matrix of bacterial biofilms is comprised primarily of polysaccharides but also contains proteins, lipids and DNA (Branda *et al*., 2005; Flemming and Wingender, 2010). The essential role of the extracellular DNA (eDNA) for stabilization of the biofilm structure was initially reported for *P. aeruginosa* (Whitchurch *et al*., 2002). The eDNA originates from a subpopulation of biofilm inhabiting bacteria, which undergoes cell lysis and releases its genomic DNA (Allesen-Holm *et al*., 2006). Further studies reported the presence of eDNA in the biofilm matrix of a wide variety of bacteria, suggesting that biofilm stabilization by eDNA is a prevalent phenomenon (Flemming and Wingender, 2010; Okshevsky and Meyer, 2013).

A unique role was assigned for eDNA in the case of the α-proteobacterium, *Caulobacter crescentus* (Berne *et al*., 2010). This bacterium is characterized by a complex life cycle that includes the production of a motile and a sessile cell at each cell division (Collier and Shapiro, 2007). The motile, swarmer cell, is characterized by a flagellum at one cell pole, whereas the sessile cell exhibits a tubular stalk structure protruding from one pole. An adhesive region present at the stalk-basis, the holdfast, allows firm attachment to surfaces, an essential step in biofilm development. The swarmer cell differentiates into a stalked cell that may subsequently settle and further divide (Fig. 3, top).

A recent study demonstrated that eDNA in biofilms of *C. crescentus* reduces the ability of its motile cells to settle (Berne *et al*., 2010). eDNA prevents attachment by interacting with the newly formed holdfast, thereby inhibiting cell adhesiveness. Purified eDNA from the extracellular matrix of *C. crescentus* has a relatively low molecular weight, mostly below 500 bp. This feature appears crucial for the inhibitory activity because intact

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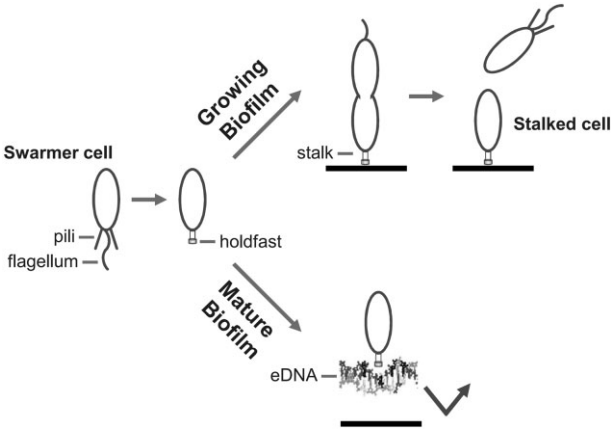


Fig. 3. eDNA masks the adhesive holdfast of *C. crescentus* and prevents progression of biofilm development. A swarmer cell develops a stalk characterized by an adhesive region, the holdfast. Upon settling on a substratum, this cell divides, giving rise to a settled stalked cell and a swarmer cell. The latter develops a stalk and a holdfast, and may attach to the surface and allow further development of the biofilm (upper scheme). The mature biofilm contains eDNA, which binds the holdfast, inhibits settling and prohibits further biofilm development. The scheme is modified from Berne and colleagues (2010).

genomic DNA of *C. crescentus* does not inhibit biofilm development, and progressive digestion of the genomic DNA increases its inhibitory activity. The inhibitory effect is specific to *C. crescentus* DNA and is not a general property of DNA or other negatively charged polymers. Cell death within the biofilm is correlated with the amount of eDNA. Taken together, the following model was suggested (Berne *et al.*, 2010): cell lysis within an existing biofilm supplements the matrix with eDNA at levels sufficient for binding to the holdfast of newly formed cells, inhibiting their attachment and thereby preventing further progression of biofilm development (Fig. 3, bottom). This self-inhibitory mechanism allows spreading of the newly formed cells, a process that may be advantageous in situations in which the biofilm habitat was extensively exploited. Given that the concentration of eDNA, the key component of the inhibitory mechanism, correlates with cell death and lysis, a gradual increase in inhibition may occur under adverse conditions. Such dependence on cell death results in an inhibitory process that reflects continuing deterioration of the biofilm habitat and, consequently, gives rise to increasing numbers of dispersing cells.

In conclusion, this mechanism is different from previously described processes of biofilm dispersal because the preformed biofilm is not affected; rather, cell progeny are prohibited from inhabiting the biofilm. It remains to be determined if eDNA is relevant for shielding adhesion molecules in additional cases [e.g. the unipolar polysaccharide produced by the plant pathogen, the α-proteobacterium *Agrobacterium tumefaciens* (Xu *et al.*, 2013)].

Biofilm inhibition by environmental cues

In the previous sections, we described cases in which the bacterium self-produces extracellular compound(s) that mitigate biofilm formation, either directly or by eliciting a cellular response inhibiting biofilm development. We now discuss several examples in which environmental cues trigger a cellular response resulting in biofilm inhibition, focusing on the effect of nutrients and light.

Biofilm inhibition by glucose. Glucose starvation leads to reduction in biofilm development by *V. cholerae* via the phosphoenolpyruvate phosphotransferase system (PTS; Houot and Watnick, 2008). Glucose phosphorylation concomitant to its uptake results in accumulation of the dephosphorylated form of $EIIA^{Gluc}$ protein, leading to biofilm formation (Fig. 4, left panel). In contrast, under glucose starvation, components of the PTS are fully phosphorylated. Thus, EI in its phosphorylated form decreases biofilm formation (Fig. 4, right panel).

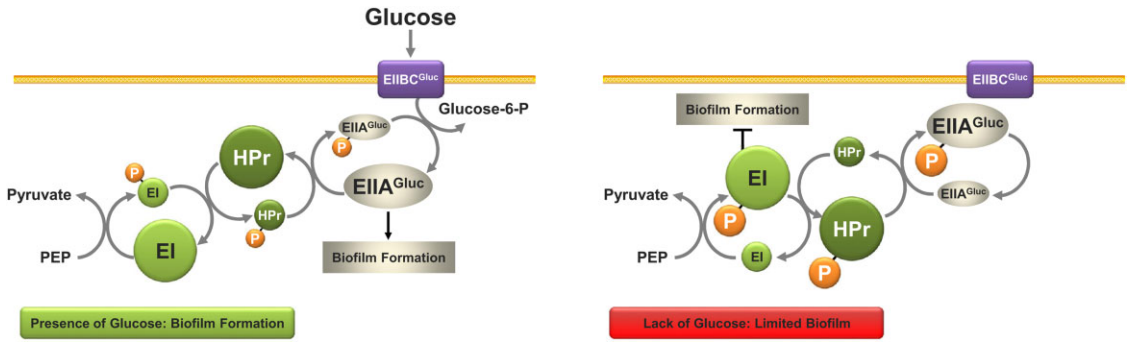


Fig. 4. Glucose starvation results in biofilm inhibition in *V. cholerae*. Glucose uptake and phosphorylation by the PTS (left panel) results in accumulation of the dephosphorylated form of the $EIIA^{Gluc}$ protein, which allows biofilm development. Under glucose starvation (right panel), the EI protein of PTS accumulates in its phosphorylated form, limiting biofilm formation. The scheme is modified from Houot and Watnick (2008). PEP, phosphoenolpyruvate.

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Biofilm inhibition by iron, zinc or phosphate. Iron concentration is a critical signal modulating bacterial biofilm development. Studies of *P. aeruginosa* revealed that iron chelation by lactoferrin prevents differentiation into towering pillars and mushroom-shaped biofilms, typical of this bacterium, allowing the formation of thin-layer biofilms (Singh *et al*., 2002). In contrast, cells mutated in the pivotal iron regulator, Fur (ferric uptake regulator), form the highly structured biofilms even in the presence of lactoferrin. These data indicate that Fur serves as a repressor of biofilm formation under iron-limiting conditions (Banin *et al*., 2005). The presence of relatively high iron concentrations (100 μM $FeCl_3$), however, prevents release of eDNA and suppresses structural biofilm development in *P. aeruginosa* (Yang *et al*., 2007). Thus, in this organism, a moderate iron level is required for biofilm development, whereas very low or high iron concentrations prevent development of highly structured biofilms.

A recent study revealed that biofilm formation in *E. coli* is governed by zinc availability via modulation of cyclic di-GMP (c-di-GMP) levels (Zahringer *et al*., 2013). c-di-GMP is a central signalling molecule governing a large array of bacterial responses; elevated levels of this second messenger promote biofilm formation (Boyd and O'Toole, 2012; Povolotsky and Hengge, 2012; Sondermann *et al*., 2012; Guttenplan and Kearns, 2013; Romling *et al*., 2013). Cellular c-di-GMP concentration is determined by the rate of its synthesis by diguanylate cyclases and degradation by phosphodiesterases. Structural analyses of DgcZ (diguanylate cyclase sensing zinc) indicated that zinc binding locks the dimerization domain of this zinc sensor, thereby preventing c-di-GMP production and reducing biofilm formation (Zahringer *et al*., 2013).

Biofilm inhibition by regulation of c-di-GMP levels is also demonstrated by *Pseudomonas fluorescens* Pf0-1 under phosphate limitation. Low phosphate sensed by the PhoR/Pst complex activates PhoR kinase activity, which in turn phosphorylates the response regulator, PhoB. This activates transcription of *rapA*, encoding a phosphodiesterase that degrades c-di-GMP. When c-di-GMP is not bound to LapD, this membrane protein cannot interact with LapG, and the latter cleaves the N-terminus of LapA, releasing this adhesin from the cell surface. Altogether, this mechanism prevents firm cell attachment to the substratum, thereby limiting biofilm development under low phosphate concentrations (Monds *et al*., 2007; Newell *et al*., 2009; 2011).

Biofilm inhibition by light. Light serves an important regulatory role in photosynthetic organisms. Intriguingly, recent bioinformatic approaches demonstrated the wide occurrence of genes putatively encoding photoreceptors in non-photosynthetic organisms, as well, lending support to the hypothesis that the regulatory function of light is not restricted to phototrophs (Van der Horst *et al*., 2007; Gomelsky and Hoff, 2011; Losi and Gartner, 2011). Below, we describe three studies suggesting involvement of light in biofilm mitigation.

The Gram-negative opportunistic human pathogen, *Acinetobacter baumannii*, forms biofilms in darkness, whereas blue light diminishes biofilm development. This phenotype is dependent on the gene, *blsA*, which encodes a protein with an N-terminus blue-light-sensing-using flavin (BLUF) domain. Purified BlsA exhibits spectral properties typical of BLUF photoreceptors, providing further support for the involvement of blue light in modulation of biofilms (Mussi *et al*., 2010). Extension of this study to additional species of the genus *Acinetobacter*, however, demonstrated that in contrast to *A. baumannii*, blue light promotes biofilm development in these bacteria (Golic *et al*., 2013). Thus, different *Acinetobacter* species employ blue light regulation of biofilms, either for limitation or activation, depending on the particular bacterium.

An additional study implicates a blue light responsive pathway in the purple photosynthetic bacterium, *Rhodopseudomonas palustris* (Kanazawa *et al*., 2010). *In vitro* characterization demonstrated that upon blue light illumination, PapB, which contains a BLUF domain, enhances the phosphodiesterase activity of PapA, which in turn degrades c-di-GMP. Because, in general, increased levels of this second messenger stimulate biofilm development, it was suggested that PapB and PapA limit biofilm development under blue light. Deletion of either *papA* or *papB* resulted in increased biofilm formation upon blue light illumination compared with dark conditions, providing further support for a blue light inhibitory pathway. Wild-type cells, however, also exhibit elevated biofilm under blue light illumination compared with dark conditions (Kanazawa *et al*., 2010). These data suggest intricate regulation of biofilms in *R. palustris*, which also involves a pathway activated by blue light.

Idiomarina loihiensis is a deep-sea γ-proteobacterium; thus, it was surprising to find in this organism a gene putatively encoding a photoactive yellow protein (Kumauchi *et al*., 2008). The observation that this gene is located next to a gene encoding diguanylate cyclase motivated examination of the involvement of light in regulation of biofilm development (Van der Horst *et al*., 2009). The absence of genetic tools dictated the use a pharmacochemical approach employing *cis*- and *trans*-locked chromophores mimicking light and dark states of the photoreceptor respectively. The *cis*-locked analogue reduced biofilm formation, while application of the *trans*-locked analogue enabled biofilm formation. These results, which support light-regulated biofilm-inhibition in *I. loihiensis*, suggest that at times, this bacterium reaches the upper

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layers of the water column and is exposed to light. Under these circumstances, planktonic growth is favoured, whereas in the deep sea, biofilm is the preferred mode of growth (Van der Horst *et al*., 2009).

It should be noted that activation of biofilm formation by light was also reported (Tschowri *et al*., 2009; Gomelsky and Hoff, 2011). Given the widespread presence of photoreceptors in heterotrophic bacteria, it is conceivable that the examples given above are only the tip of the iceberg, and light regulation of biofilms is a more common mechanism than previously believed.

Concluding remarks and prospective

Bacteria exhibit diverse mechanisms that reduce biofilm development. In some cases, bacterially produced extracellular substances, e.g. small quorum-sensing signal molecules, high-molecular weight polysaccharides or eDNA, are key components of the inhibitory process. The dependence on extracellular compounds and the fact that the producing bacteria as well as their neighbouring siblings are affected, support the suggestion that these inhibitory mechanisms evolved to provide regulation at the communal rather than at the single cell level. Nevertheless, particular responses do appear to occur at the single cell level (e.g. inhibitory responses triggered by blue light or glucose concentration). Future studies may uncover an additional layer of regulation that will provide support for 'multicellularity' in these cases, as well.

Some of the mechanisms described here are represented by only a single example (e.g. inhibition of adhesion by eDNA) or by very few reports (e.g. biofilm limitation by blue light). However, considering the frequent occurrence of eDNA in bacterial biofilms, and the high prevalence of genes encoding putative light sensors, it is likely that these pathways will also be shown to represent general mechanisms of biofilm regulation.

As described, numerous studies identified bacterially produced substances that cause the dispersal of existing biofilms. Many of these compounds result in *a priori* inhibition of biofilm development when exogenously added to planktonic cultures. It remains to be seen whether, under particular conditions, these molecules are natively produced at the planktonic stage of growth.

Following characterization of the inhibitory mechanisms in the context of a single species, it will be intriguing to examine the function of these processes in multispecies cultures. This will unravel further complexity in cases where molecules secreted by a specific species are capable of cross-activation or cross-inhibition of another species. Additional intricacy may be provided by the ability of a particular species to degrade or chemically modify compounds secreted by a competing species sharing the same growth niche. The information gleaned from these studies will provide additional insight into self-inhibitory processes as well as inhibition of competing species, and should be applicable for designing new strategies to minimize damage resulting from detrimental biofilms.

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