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Minireview

Quorum sensing in *Pseudomonas aeruginosa* biofilms

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Summary

In nature, the bulk of bacterial biomass is believed to exist as an adherent community of cells called a biofilm. *Pseudomonas aeruginosa* has become a model organism for studying this mode of growth. Over the past decade, significant strides have been made towards understanding biofilm development in *P. aeruginosa* and we now have a clearer picture of the mechanisms involved. Available evidence suggests that construction of these sessile communities proceeds by many different pathways, rather than a specific programme of biofilm development. A cell-to-cell communication mechanism known as quorum sensing (QS) has been found to play a role in *P. aeruginosa* biofilm formation. Because both QS and biofilms are impacted by the surrounding environment, understanding the full involvement of cell-to-cell signalling in establishing these complex communities represents a challenge. Nevertheless, under set conditions, several links between QS and biofilm formation have been recognized, which is the focus of this review. A role for antibiotics as alternative QS signalling molecules influencing biofilm development is also discussed.

Introduction

In nature, bacteria commonly exist as a sessile, slime-encased community of cells forming what is known as a biofilm. The physical structure of biofilms is highly variable ranging from flat, thin-layered mats to complex stalk- and mushroom-like constructions (Figs 1 and 2). In some instances, biofilms are populated by a single species whereas in others, the inhabitants are comprised of a diverse microbial array. For residents of a biofilm, the communal lifestyle offers considerable advantages over the planktonic mode of growth. For example, bacteria living as a biofilm are significantly more tolerant to antibiotics and biocides and they enjoy shelter from environmental stresses, including attack by the host immune response. Furthermore, the close proximity of cells facilitates horizontal gene transfer and sharing of metabolic by-products within the biofilm community. The elaborate nature of bacterial biofilms has led some scientists to make comparisons with multicellular structures reminiscent of higher organisms. With this in mind, it is not surprising that construction of these complex communities requires coordinated interaction between the bacterial inhabitants. A cell-to-cell communication mechanism known as quorum sensing (QS) has been found to play an important role in biofilm formation. The QS systems rely on self-generated signalling molecules to coordinate gene expression in response to population density. The majority of signalling molecules identified thus far can be classified into three main groups: acylhomoserine lactones (AHLs), oligopeptides and the LuxS/autoinducer 2 (Keller and Surette, 2006). Nevertheless, the types of chemicals associated with cell-to-cell signalling represent an ever-expanding collection of molecules that are structurally quite diverse.

The Gram-negative bacterium *Pseudomonas aeruginosa* has become a model organism for independently studying these two social phenomena, namely QS and biofilm formation. In a seminal investigation in 1998, Davies and co-workers discovered a link between them; QS was reported to be required for elaboration of mature, differentiated *P. aeruginosa* biofilms. Since that time exhaustive effort has been directed towards uncovering the mechanism(s) by which QS regulates biofilm production. One of the end goals being that elucidating the pathways of biofilm development will make it possible to control their formation. The aim of this review is to discuss the role of *P. aeruginosa* cell-to-cell signalling in establishing these complex communal structures.

Quorum sensing in *P. aeruginosa*

Pseudomonas aeruginosa has two AHL-based QS systems; Las and Rhl. The Las system is comprised of the transcriptional regulator LasR and its cognate AHL signal,

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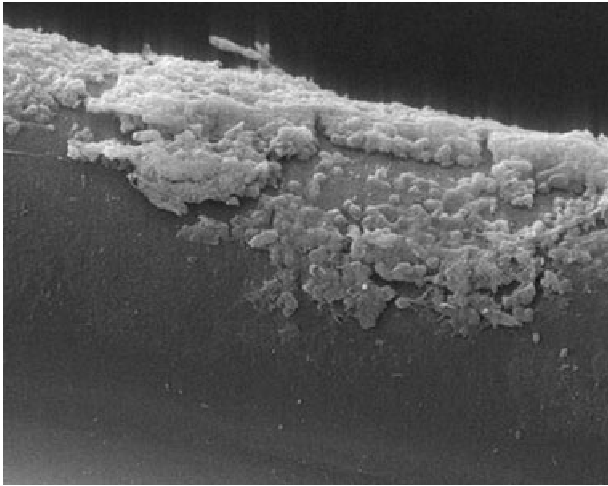

Fig. 1. *Pseudomonas aeruginosa* biofilm formed on a suture. Photograph courtesy of G.A. O'Toole, Dartmouth Medical School.

N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), synthesized by the AHL synthase LasI (Gambello and Iglewski, 1991; Passador *et al.*, 1993). Similarly, the Rhl system is comprised of RhlR together with its cognate AHL, *N*-butyryl-L-homoserine lactone (C4-HSL), synthesized by the RhlI AHL synthase (Ochsner *et al.*, 1994a; Latifi *et al.*, 1995). In addition to 3-oxo-C12-HSL and C4-HSL, *P. aeruginosa* produces a third signalling molecule, 2-heptyl-3-hydroxy-4(1*H*)-quinolone, called *Pseudomonas* Quinolone Signal (PQS) (Pesci *et al.*, 1999). Structural genes for PQS production have been identified (*pqsABCDH*) together with a transcriptional regulator (*pqsR*) and the response effector (*pqsE*) (Gallagher *et al.*, 2002).

Three independent studies have shown that up to 11% of the *P. aeruginosa* genome is subject to AHL-dependent regulation (Whiteley *et al.*, 1999; Schuster *et al.*, 2003; Wagner *et al.*, 2003). The challenge thus becomes dissecting which QS-controlled genes influence biofilm development. In the initial report by Davies and colleagues (1998), the Las QS system was found to be essential for creation of mature, differentiated biofilms. In a flow-chamber set-up, stalk-like structures with intervening water channels composed the PAO1 biofilm. In sharp contrast, the *lasI*$^-$ biofilm was flat and undifferentiated. Upon exposure to the detergent sodium dodecyl sulfate (SDS), the *lasI*$^-$ biofilm quickly dispersed from the substratum, whereas the wild-type biofilm remained intact. So in addition to being structurally altered, the mutant biofilm was functionally impaired in its ability to resist biocidal agents. Because no discernable differences were observed between the *rhlI*$^-$ and PAO1 biofilms, it was concluded that the Rhl QS was not involved (Davies *et al.*, 1998). Since this inaugural report, several groups have published findings supporting a role for QS, including the Rhl QS system, in *P. aeruginosa* biofilm formation (Hentzer *et al.*, 2002; Allesen-Holm *et al.*, 2006; Sakuragi and Kolter, 2007; Barken *et al.*, 2008). However, in some cases a link has not been established (Heydorn *et al.*, 2002; Purevdorj *et al.*, 2002; Schaber *et al.*, 2007). The most plausible explanation for these discrepancies is that different experimental parameters have a significant impact on biofilm formation, discussed in detail below.

Biofilm matrix

The extracellular polymeric substance (EPS) that forms the cohesive matrix surrounding cells in the biofilm typically consists of polysaccharide, DNA and proteins. In *P. aeruginosa*, five gene clusters have been identified that are believed to function in exopolysaccharide synthesis, including the *alg* biosynthetic genes (PA3540–PA3551), the *psl* (PA2231–PA2245) and *pel* (PA3058–PA3064) operons and two other gene clusters (PA1381–PA1391 and PA3552–PA3558). Thus far, only the *pel* biosynthetic operon has been definitively identified as being subject to QS regulation (Sakuragi and Kolter, 2007). The *pel* gene cluster (*pelABCDEFG*) encodes proteins responsible for the production of glucose-rich biofilm exopolysaccharide (Friedman and Kolter, 2004). This polysaccharide imparts a wrinkled colony phenotype and is essential for surface-associated biofilm or pellicle formation (Friedman and

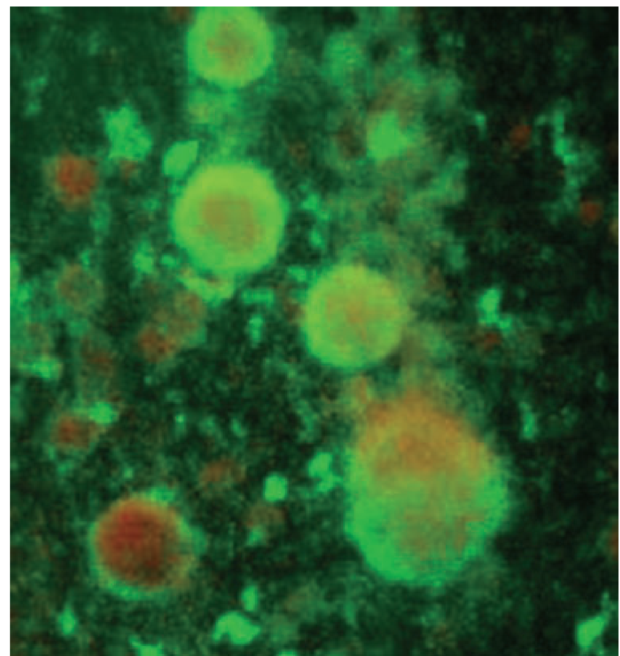

Fig. 2. *Pseudomonas aeruginosa* biofilm established in a flow-cell system. Stalk-like structures with intervening water channels can be seen in the mature biofilm. Bacteria expressing GFP were stained with propidium iodide; therefore, green bacteria represent live cells while reddish-coloured cells are dead. Photograph courtesy of G.A. O'Toole, Dartmouth Medical School.

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Kolter, 2004). Analysis of Las QS mutants revealed a significant reduction in solid surface-associated biofilm and air-liquid pellicle formation (Sakuragi and Kolter, 2007). It was discovered that the Las QS system and to a lesser extent the Rhl system is involved in transcription of the *pel* biosynthetic genes.

In addition to polysaccharides, extracellular DNA is an important component of the *P. aeruginosa* biofilm matrix (Whitchurch *et al.*, 2002a; Nemoto *et al.*, 2003). When relatively young PAO1 biofilms ($\leq$ 60 h) grown in flow-cell chambers were exposed to DNase I, they completely dissolved; whereas older biofilms were only nominally affected (Whitchurch *et al.*, 2002a). These findings suggest that early on in biofilm development, extracellular DNA is a key component holding cells together. But as the biofilm matures, other substances replace DNA as the primary cohesive factor. In a subsequent study, it was reported that extracellular DNA in *P. aeruginosa* biofilms is generated from the lysis of a small population of cells by two different pathways (Allesen-Holm *et al.*, 2006). One pathway is linked to QS and results in the release of larger amounts of DNA; the second QS-independent pathway liberates a basal level of extracellular DNA (Allesen-Holm *et al.*, 2006). Biofilms formed by *lasIrhlI* and *pqsA* QS mutants exhibited reduced extracellular DNA levels and increased susceptibility to SDS treatment, suggesting that extracellular DNA helps stabilize the biofilm matrix. Furthermore, spatial analysis revealed distinct patterns of DNA organization. For example in 2 day glucose-grown PAO1 biofilms, the DNA was most highly concentrated on the surface of the microcolonies, whereas in more mature biofilms, the extracellular DNA was shown to accumulate on the outer parts of the stalks, particularly at the junction between the stalk and the mushroom cap (Allesen-Holm *et al.*, 2006). Previously, de Kievit and colleagues (2001) discovered that *lasI* and *rhlI* expression were maximal in the portion of the biofilm closest to the substratum. This is consistent with the observation that the greatest difference between the QS mutants and the parent in the amount of extracellular DNA produced occurs near the substratum (Allesen-Holm *et al.*, 2006). In addition, the *pqsA* mutant biofilm was thin and flat with very little extracellular DNA (Allesen-Holm *et al.*, 2006). Yang and colleagues (2007) reported that a *pqsA–gfp* reporter fusion was expressed primarily in cells on the outer layer of stalks produced in differentiated biofilms. Thus, these gene expression studies support findings regarding the distribution of extracellular DNA and the role of QS in DNA release.

Rhamnolipids

Rhamnolipids are amphipathic glycolipids that can act as biosurfactants. *Pseudomonas aeruginosa* rhamnolipid production occurs through the *rhlAB* operon and *rhlC*; all three of these genes are under QS control (Ochsner *et al.*, 1994a,b; Ochsner and Reiser, 1995; Pearson *et al.*, 1997; Rahim *et al.*, 2001). A number of studies have revealed that rhamnolipids play multiple roles in the establishment and maintenance of *P. aeruginosa* biofilms. Microscopic analysis of biofilms formed by *P. aeruginosa* on solid surfaces has revealed cells embedded in an EPS matrix that contains open channels (Fig. 2). These channels or void spaces presumably facilitate access to nutrients and oxygen and enable removal of waste products (Lawrence *et al.*, 1991). Rhamnolipids have been shown to be necessary for maintaining the open channel structures surrounding microcolonies (Davey *et al.*, 2003). While the biofilm formed by a *rhlA* mutant, deficient in rhamnolipid production, was flat and undifferentiated, that of the wild type was characterized by stalk-like structures with intervening water channels (Davey *et al.*, 2003). It was concluded that rhamnolipids are not required for initial microcolony or channel formation; however, once established, rhamnolipids play an integral role in keeping the channels open. Another group confirmed that an *rhlA* mutation results in biofilms that are flat and relatively homogeneous (Pamp and Tolker-Nielsen, 2007). However, in contrast to what Davey and colleagues reported, Pamp and Tolker-Nielsen (2007) found that the *rhlA*⁻ strain was deficient in microcolony formation. To address this inconsistency, the *rhlA* mutation was moved into several different PAO1 isolates. In all cases, the *rhlA* mutants showed aberrant microcolony development. It was proposed that the *rhlA*⁻ strain used by Davey and co-workers harbours additional mutations, allowing it to form microcolonies in the absence of rhamnolipid (Pamp and Tolker-Nielsen, 2007). A second *rhlA*-mediated difference in biofilm formation was reported in this study (Pamp and Tolker-Nielsen, 2007). When grown in flow-cell chambers with glucose as a carbon source, the caps formed by the *rhlA* mutants were significantly smaller than those of the wild type. Thus it appears that rhamnolipid production facilitates mushroom cap formation in *P. aeruginosa* biofilms (Pamp and Tolker-Nielsen, 2007). Consistent with this notion, expression of a *rhlA–gfp* fusion was found to be maximal in the stalks of a developing biofilm with only modest GFP fluorescence visible in the mushroom caps (Lequette and Greenberg, 2005). It was postulated that rhamnolipid synthesis in the growing stalks may facilitate migration of a motile subpopulation of cells up the stalk, resulting in mushroom cap formation.

Rhamnolipids are also believed to be involved in detachment of cells from the biofilm (Schooling *et al.*, 2004; Boles *et al.*, 2005). Detachment of bacteria differs from shear force-generated sloughing (Picioreanu *et al.*, 2001) as detachment can be stimulated by environmental triggers allowing a transition between biofilm and plank-

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tonic lifestyles (Sauer *et al*., 2004). In a study done by Boles and colleagues (2005), a hyper-detachment mutant was found to overexpress rhamnolipids. Inactivation of the *rhlAB* genes in this mutant abolished accelerated detachment. In biofilms formed by the wild type, hollow cores could be observed within the centre of the biofilm clusters but only after 10–12 days of biofilm maturation. Conversely, induction of plasmid-borne *rhlAB* genes in wild-type cells caused central hollowing in much younger 3-day-old biofilms. Addition of exogenous surfactants, including rhamnolipid and SDS, could also induce this hollowing effect in established biofilms (Boles *et al*., 2005).

Finally, the involvement of rhamnolipids in the formation of these sessile structures was supported by the findings of Morici and colleagues (2007). In this study, the *P. aeruginosa* virulence regulator AlgR was investigated for its role in biofilm development (Morici *et al*., 2007). Using static and continuous-culture systems, an *algR* mutant was impaired in its ability to establish a biofilm (Whitchurch *et al*., 2002b; Morici *et al*., 2007). Transcriptional profiling of biofilm-grown PAO1 revealed that *rhlA* and *rhlB* were the two most highly repressed genes in the AlgR regulon (Morici *et al*., 2007). These researchers demonstrated that AlgR binds directly to the promoter regions of *rhlA* and *rhlI*. Moreover, the *algR* deletion strain produced higher amounts of rhamnolipid and C4-HSL in biofilms but not in planktonic cultures, indicating that this regulation is biofilm-specific. It was proposed that AlgR repression of *rhlAB* and the Rhl QS is critical for normal biofilm maturation (Morici *et al*., 2007).

Collectively, these findings suggest that rhamnolipids influence multiple facets of *P. aeruginosa* biofilm formation, including: (i) microcolony formation, (ii) maintenance of open channels, (iii) mushroom cap formation and (iv) detachment of cells from the biofilm. Because rhamnolipids are subject to QS control, this is another way in which intercellular communication affects formation of these sessile communities.

Carbon source, bacterial migration and *P. aeruginosa* biofilm formation

In liquid environments, *P. aeruginosa* swims via a single-polar flagellum. On solid surfaces, *P. aeruginosa* is capable of two types of surface-associated movement; twitching and swarming motility. Twitching is mediated by type IV pili while swarming is dependent upon QS, a functional flagellum, biosurfactant production and in some instances type IV pili (Köhler *et al*., 2000; Mattick, 2002; Déziel *et al*., 2003). Quorum sensing is believed to control swarming through rhamnolipid production, as the *rhlAB* genes are QS-regulated (Ochsner *et al*., 1994a). Evidence has been presented supporting a role for bacterial migration in both early and late stages of biofilm development that is dependent upon pili, flagella and rhamnolipid. The contribution of each of these, however, is strongly dependent on nutritional conditions.

One of the first investigations of genes involved in *P. aeruginosa* biofilm initiation revealed that flagella play an important role in attachment to a solid surface in glucose minimal media (O'Toole and Kolter, 1998). Furthermore, mutants defective in type IV pilus biogenesis were able to attach, but they were unable to form the characteristic microcolonies observed with the *P. aeruginosa* wild type (O'Toole and Kolter, 1998). Type IV pili-biogenesis was later found to be regulated by the catabolite repressor control protein, providing a connection between nutritional cues and biofilm development (O'Toole *et al*., 2000). Swarming motility has also been implicated in early stages of *P. aeruginosa* biofilm establishment (Shrout *et al*., 2006). Under conditions that promote swarming motility (succinate and glutamate), cells continuously move over the surface resulting in a flat, uniform biofilm. Under swarm-limiting conditions (glucose), the biofilm is punctuated by microcolonies. These findings led to the proposal that the magnitude of swarming motility during the initial stages of biofilm development ultimately affects whether the biofilm is flat and homogenous or highly ordered (Shrout *et al*., 2006). It was also discovered that QS control of swarming was dependent on carbon source. Biofilms formed by QS mutants were quite different from those of the PAO1 parent when grown on succinate, but not glucose or glutamate. Under succinate conditions, the mutant biofilms contained microcolonies; whereas the wild-type biofilm was flat and uniform (Shrout *et al*., 2006). The presence of microcolonies suggested that the QS mutants exhibited decreased swarming motility on succinate media, and this was confirmed to be the case. At present, the means by which carbon source controls swarming motility has yet to be resolved. Because no differences in signal production were detected, the nutritional environment did not appear to affect QS *per se* (Shrout *et al*., 2006). It should be mentioned here that other studies have shown that growth conditions influence both timing and overall expression of the *las* and *rhl* QS systems (Duan and Surette, 2007).

The impact of carbon source can also be seen later in development when the mature biofilm architecture is being established. In flow channels containing glucose minimal media, *P. aeruginosa* PAO1 forms heterogeneous biofilms characterized by mushroom-like structures with intervening water channels, whereas cells grown in minimal citrate, benzoate or casamino acids medium produced a flat uniform biofilm (Heydorn *et al*., 2002; Klausen *et al*., 2003a,b). Bacterial cells exhibit extensive twitching motility in citrate minimal media and this is hypothesized

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to mediate a flat biofilm phenotype (Klausen *et al.*, 2003b). The formation of mushroom-shaped structures under glucose conditions was proposed to follow a sequential process. Time-lapse confocal laser scanning microscopy of biofilms formed by colour-coded combinations of *P. aeruginosa* wild type and *pilA* mutants (deficient in pilus production) revealed that a non-motile subpopulation develops into stalks, which are then capped by a migrating group of motile cells (Klausen *et al.*, 2003a). These initial findings indicated that mushroom cap formation required type IV pili and involved bacterial migration; consequently, type IV-pilus-mediated motility was thought to be the means of bacterial migration (Klausen *et al.*, 2003a). However, it was later discovered that flagellar-driven motility and chemotaxis, rather than pilus-mediated motility, are involved in cap formation (Barken *et al.*, 2008). The requirement for type IV pili in this process was proposed to be linked, via high-affinity binding, to extracellular DNA (Barken *et al.*, 2008). Because *P. aeruginosa* QS mutants liberate significantly less DNA in the biofilm matrix, a *pilAlasRrhlR* mutant was created. Using time-lapse confocal laser scanning microscopy and colour-coded combinations of wild type/*pilAlasRrhlR* and wild type/*pilA* mixed biofilms, the wild-type cells were found capping stalks formed by the *pilA* but not the *pilAlasRrhlR* strain (Barken *et al.*, 2008). So QS and DNA release appear to be important for development of mushroom cap structures in *P. aeruginosa* biofilms. It was acknowledged that QS-controlled factors other than DNA might also be involved (Barken *et al.*, 2008). For example, a *pilAlasRrhlR* mutant would be deficient in rhamnolipid production and this surfactant was previously shown to facilitate bacterial migration and establishment of mushroom caps (Pamp and Tolker-Nielsen, 2007). Regardless of the exact mechanism, these findings indicate that QS-regulated factors combined with flagellum-driven motility and chemotaxis, type IV pili but not pilus-mediated motility all play a role in cap formation (Barken *et al.*, 2008).

Effect of iron on QS and biofilm formation

It is well established that *P. aeruginosa* QS gene expression is enhanced under Fe-limiting conditions (Bollinger *et al.*, 2001; Kim *et al.*, 2005; Bredenbruch *et al.*, 2006; Duan and Surette, 2007) and several groups have reported a connection between iron availability and biofilm formation (Singh *et al.*, 2002; Singh, 2004; Banin *et al.*, 2005; Musk *et al.*, 2005; Yang *et al.*, 2007). In a study done by Yang and colleagues (2007), the effects of iron on DNA release and biofilm formation were investigated. Analysis of static biofilms formed in microtitre plates revealed that biofilm formation, DNA release and *pqs* expression were maximal under low-iron conditions (5 μM) and decreased with increasing iron concentrations up to 100 μM. In a flow-cell system, mushroom-shaped structures were observed in the *P. aeruginosa* biofilms under iron-limiting conditions (1 μM). Under high-iron conditions (100 μM), the biofilm contained less biomass and did not develop stalk-like structures. Furthermore, less extracellular DNA and reduced *pqsA* expression were observed under these conditions. It was concluded that low iron concentrations promote *P. aeruginosa* biofilm development, and this is mediated by upregulation of *pqs* genes and concomitant release of high levels of extracellular DNA (Yang *et al.*, 2007).

Patriquin and colleagues (2008) found a different correlation between available iron and biofilm biomass in a static plate assay. In this case, Fe concentrations below 8 μM markedly reduced biofilm yields. Flow-cell analysis revealed a transition from flat, uniform biofilms to highly ordered structures going from iron-deplete to iron-replete media, which also differs from previous findings (Yang *et al.*, 2007). Interestingly, a *rhlI*$^{-}$ mutant produced differentiated biofilms in iron-deplete media, suggesting that the Rhl QS system is somehow involved. These researchers discovered that wild-type bacteria growing under iron-limiting conditions exhibited enhanced twitching motility (Patriquin *et al.*, 2008). Addition of spent culture supernatants from cells grown with low iron, but not purified C4-HSL, stimulated twitching motility in cells grown on iron-replete media (typically low twitching). Consequently, it was suggested that an iron-regulated, Rhl-dependent extracellular product was responsible for enhanced twitching and reduced biofilm formation in response to iron limitation. Because heat and protease treatment did not hinder the twitch-promoting properties of these supernatants, rhamnolipids were suggested as a potential candidate, but this remains speculative (Patriquin *et al.*, 2008).

The apparent discrepancies regarding the impact of iron availability on biofilm formation (Yang *et al.*, 2007; Patriquin *et al.*, 2008) might be due, at least in part, to media differences. For plate assays, AB glucose and BM2 succinate minimal media were used by Yang and colleagues (2007) and Patriquin and colleagues (2008) respectively. For the flow-cell experiments, cells were grown in FAB glucose supplemented with $FeCl_3$ in one instance (Yang *et al.*, 2007), while in the other, 1% TSB containing lactoferrin to induce iron limitation was used (Patriquin *et al.*, 2008). Because carbon source affects biofilm formation, different growth conditions significantly hamper comparison of findings, as is the case here.

The hydrodynamic environment impacts QS and biofilm formation

The hydrodynamic environment in which biofilms are found can vary greatly. Conditions range from turbulent

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flow, for instance biofilms formed on catheters or rocks in a river, to virtually no flow, such as biofilms established in the lungs of cystic fibrosis (CF) patients. In one study, the effect of hydrodynamic stress on *P. aeruginosa* was investigated using a low-shear suspension culture device called the rotating wall vessel (RWV) (Crabbé *et al*., 2008). Growth of *P. aeruginosa* in the RWV was proposed to mimic conditions in the CF lung (Crabbé *et al*., 2008). Upon addition of a ceramic bead to the RWV, a higher-shear environment is created, and so the impact of low- and high-shear forces on bacterial physiology can be assessed. In a low-shear environment, *P. aeruginosa* generated a suspension biofilm, whereas high-shear conditions caused surface-adherent biofilms to form (Crabbé *et al*., 2008). Gene expression analysis revealed that two genes in the *psl* biosynthetic operon (*pslA* and *pslD*) were induced in a low-shear environment. Psl is a mannose-rich polysaccharide that forms part of the EPS matrix of the biofilm. In terms of QS, no differences in *las* gene expression were observed; however, *rhlI* and *rhlA* transcription was induced under low-shear conditions (Crabbé *et al*., 2008). Accordingly, rhamnolipid production was increased in this environment (Crabbé *et al*., 2008). It is of interest to note that very high levels of rhamnolipid have been reported in CF sputum (Kownatzki *et al*., 1987). Moreover Singh and colleagues (2000) found that the ratio of C4-HSL to 3-oxo-C12 production in CF sputum was increased compared with what is observed in *P. aeruginosa* broth cultures. These findings support the notion that the RWV is a good device for mimicking CF lung conditions.

In another study, the impact of hydrodynamic environment on QS induction in *P. aeruginosa* biofilms was investigated (Kirisits *et al*., 2007). Using a *P. aeruginosa* chromosomal *lasB–gfp* fusion strain, it was discovered that as the flow rate increased, so did the amount of biofilm biomass required for full QS induction. At the highest flow rate, QS was not fully induced, suggesting that under these conditions QS may not significantly impact biofilm formation (Kirisits *et al*., 2007). Indeed, Purevdorj and colleagues (2002) observed no major differences between wild-type and QS mutant biofilms under high-flow conditions.

Dentrification, QS and biofilm formation

Pseudomonas aeruginosa is a denitrifying bacterium that can grow anaerobically using molecules such as nitrite (NO_2^-) and nitrate (NO_3^-) as terminal electron acceptors. During anaerobic respiration, potentially toxic by-products called reactive nitrogen intermediates are produced. These reactive nitrogen intermediate molecules include nitric oxide (NO), peroxynitrite ($ONOO^-$) and nitrous acid (HNO_2) among others. Yoon and colleagues (2002) demonstrated that *P. aeruginosa* forms robust biofilms under anaerobic conditions, similar to what is believed to occur in the CF airway. While growing as an anaerobic biofilm, *rhl*-deficient mutants were readily killed due to toxic NO build-up; consequently, the Rhl QS system was deemed essential for anaerobic biofilm survival (Yoon *et al*., 2002). In a second study, it was observed that exposure to sublethal NO concentrations caused dispersion of existing biofilms and increased *P. aeruginosa* sensitivity to a number of antimicrobial compounds (Barraud *et al*., 2006). Finally, a link between denitrification and QS was reported by Toyofuku and colleagues (2007). The NO_3^- respiring activity of a Δ*lasI* mutant and a Δ*rhlI* mutant was 3.5-fold and 1.4-fold higher respectively, than the PAO1 parent (Toyofuku *et al*., 2007). Collectively, it appears that exposure to NO leads to reduced viability and increased dispersion of *P. aeruginosa* biofilms and that the Las and Rhl QS systems repress denitrification activity. Although the molecular mechanisms underlying the aforementioned findings have not been elucidated, a combination treatment involving NO exposure and conventional antimicrobial agents as a means of controlling persistent biofilm infections is very enticing (Barraud *et al*., 2006).

Antibiotics as alternative signalling molecules

While the spectrum of chemical molecules identified as bacterial cell-to-cell signals continues to expand, it can be assumed that we have only scratched the surface. With this in mind, these molecules should be considered at a functional level, rather than on a structural basis. For classification as a QS signal, molecules should accumulate according to population density, be released and subsequently recognized by adjacent cells, and ultimately affect gene transcription. Antibiotics are one group of molecules that fit this definition. Because the soil is rich with antibiotic-producing microbes, it has long been assumed that the primary role of antibiotics is to inhibit competitors in natural habitats. However, it has not been firmly established that antibiotic concentrations in soil are typically high enough to exert a killing effect. This has led to the idea that antibiotics have different concentration-dependent roles. At lower concentrations, antibiotics act as signalling molecules and modulate gene expression, whereas at higher concentrations, they function as inhibitors (for reviews see Davies *et al*., 2006; Fajardo and Martínez, 2008). Approaching this from another direction, some QS signals have been found to exhibit antimicrobial properties. For instance, the *P. aeruginosa las* QS signal 3-oxo-C12-HSL can inhibit Gram-positive bacteria and has cytotoxic effects (Kaufmann *et al*., 2005). The PQS QS signal showed no antibiotic activity against *Escherichia coli* and *Staphyloccocus aureus* (Pesci *et al*., 1999);

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however, this quinolone derivative does belong to the 'pyo' family of antibiotics produced by *P. aeruginosa* (Hays *et al.*, 1945; Wells, 1952). So whether a microbial compound acts as an antibiotic or a signalling molecule in any given environment may largely depend on its concentration and the surrounding microbiota.

Pseudomonas aeruginosa secretes phenazine antibiotics, a group of heterocyclic, redox-active compounds that are toxic to both prokaryotes and eukaryotes (Mazzola *et al.*, 1992). Dietrich and colleagues (2006) demonstrated that pyocyanin was one of the most abundant phenazines found in *P. aeruginosa* culture supernatants. To understand how *P. aeruginosa* reacts to these toxic molecules, cultures exposed to pyocyanin were subjected to gene expression analysis. Pyocyanin caused increased transcription of a set of genes designated the 'PYO stimulon' (Dietrich *et al.*, 2006). The most upregulated genes within this stimulon include *mexGHI-opmD*, which encodes an efflux pump and PA2274, a putative flavin-dependent monooxygenase. Interestingly, the QS signalling molecule PQS was previously shown to be required for expression of *phz* (and therefore pyocyanin) as well as *mexGHI-opmD* and PA2274 (Gallagher *et al.*, 2002; Déziel *et al.*, 2004; 2005). It was concluded that PQS controls pyocyanin production, and pyocyanin in turn regulates *mexGHI-opmD* and PA2274 expression (Dietrich *et al.*, 2006). In this manner pyocyanin is functioning as both an antibiotic and a QS signal. Whether pyocyanin production plays any role in *P. aeruginosa* biofilm formation was not investigated. However, a connection between phenazines and biofilm production has been established in another pseudomonad (Maddula *et al.*, 2006; 2008). *Pseudomonas chlororaphis* 30-84 is a biocontrol strain that produces two phenazines, phenazine-1-carboxylic acid (PCA) and 2-hydroxyphenazine (2-OH-PCA). Evidence suggests that both QS and phenazines are involved in biofilm formation (Maddula *et al.*, 2006). In addition, it was discovered that altering the PCA:2-OH-PCA phenazine ratio affects multiple aspects of biofilm development, including initial attachment, the mature biofilm structure and dispersal (Maddula *et al.*, 2008). How exactly phenazines mediate the observed changes in biofilm development awaits determination.

Looking at the influence of other antibiotics on this process, subinhibitory concentrations of tobramycin, tetracycline and norfloxacin have been reported to induce biofilm formation in *P. aeruginosa* (Linares *et al.*, 2006). Tobramycin stimulated swimming and swarming motility, whereas tetracycline had no effect and ciprofloxacin decreased both forms of motility (Linares *et al.*, 2006). Presumably some mechanism(s) other than motility must account for the increased biofilm formation in the presence of antibiotics. Tobramycin-associated biofilm induction in *P. aeruginosa* has also been reported by Hoffman and colleagues (2005). In this case, the gene associated with biofilm induction was identified and designated *arr* (aminoglycoside response regulator). Arr is predicted to be an inner-membrane phosphodiesterase whose substrate is the second messenger cyclic di-guanosine monophosphate (Hoffman *et al.*, 2005). Although there was no difference in planktonic sensitivity, biofilms formed by the *arr* mutant were 100-fold more susceptible to tobramycin killing than the wild type. Thus, *arr* appears to be a genetic determinant of biofilm-associated aminoglycoside resistance (Hoffman *et al.*, 2005).

Taken together, these findings suggest that antibiotics may be more than weapons for destroying competing microbes. They may also act as signalling molecules enabling microbial communities to coordinate an adaptive response to an ever-changing environment. Production of both QS signals and antibiotics represents a metabolic burden to the producer. If these molecules serve multiple functions, they are more likely to be maintained against the forces of natural selection. The fact that QS signals have antimicrobial properties and antibiotics can trigger adaptive responses supports a dual role for these molecules in nature.

Concluding remarks

Over the past decade, significant strides have been made towards understanding biofilm development in *P. aeruginosa* and we now have a much clearer picture of the mechanisms involved. An underlying message that keeps emerging is that development of these sessile communities may proceed by many different pathways. Indeed, comparative transcriptome and proteome analyses of *P. aeruginosa* biofilm and planktonic cells (Whiteley *et al.*, 2001; Sauer *et al.*, 2002; Hentzer *et al.*, 2004) failed to reveal a specific programme of biofilm development. Rather, available evidence suggests that bacteria employ different mechanisms for surface colonization in response to the prevailing environmental conditions. For instance, many factors that affect biofilm architecture, including bacterial motility and the composition of the biofilm matrix, are conditionally dependent. Because both QS and biofilm formation are impacted by the surrounding environment, understanding the full involvement of cell-to-cell signalling in the formation of these complex communities is a daunting challenge. Nevertheless, under set conditions, several links between QS and biofilm formation have been established. A model of *P. aeruginosa* biofilm development is depicted in Fig. 3, with connections to QS indicated. As we continue to investigate how QS and other types of signalling, such as second messenger signals affect group behaviour, a clearer picture of exactly how these complex communal structures are created will emerge.

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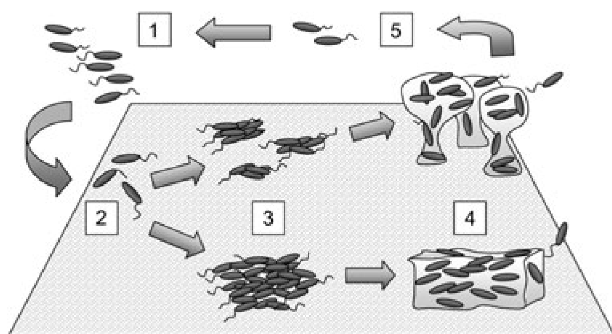

Fig. 3. *Pseudomonas aeruginosa* biofilm development. Planktonic cells (stage 1) attach onto a solid surface (stage 2) and microcolonies are formed (stage 3). Under conditions that promote bacterial migration (e.g. succinate, glutamate), cells will spread over the substratum, ultimately developing into a flat, uniform mat (stage 4). Under motility-limiting conditions (e.g. glucose), the microcolonies proliferate forming stalk- and mushroom-like structures (stage 4). At various points throughout biofilm maturation, cells can detach and resume the planktonic mode of growth (stage 5). QS-controlled rhamnolipid production impacts microcolony formation (stage 3), maintenance of open channels (stage 4), mushroom cap formation (stage 4) and dispersion from the biofilm (stage 5). In addition, production of Pel polysaccharide and DNA release, which are both important for the EPS matrix (stages 3 and 4), are under QS control.

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