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REVIEW ARTICLE

Biofilm and motility in response to environmental and host-related signals in Gram negative opportunistic pathogens

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Summary

Most bacteria can switch between a planktonic, sometimes motile, form and a biofilm mode, in which bacterial cells can aggregate and attach to a solid surface. The transition between these two forms represents an example of bacterial adaptation to environmental signals and stresses. In 'environmental pathogens', namely, environmental bacteria that are also able to cause disease in animals and humans, signals associated either with the host or with the external environment, such as temperature, oxygen availability, nutrient concentrations etc., play a major role in triggering the switch between the motile and the biofilm mode, via complex regulatory mechanisms that control flagellar synthesis and motility, and production of adhesion factors. In this review article, we present examples of how environmental signals can impact biofilm formation and cell motility in the Gram negative bacteria *Pseudomonas aeruginosa*, *Escherichia coli* and in the *Burkholderia* genus, and how the switch between motile and biofilm mode can be an essential part of a more general process of adaptation either to the host or to the external environment.

Introduction

In order to efficiently colonize environmental niches, often characterized by very diverse features, micro-organisms have developed various adaptation strategies. Indeed, several bacteria associated with humans and warm-blooded animals, including pathogenic species, are at the same time 'environmental' micro-organisms, namely, they can survive, or even thrive, in soil, water or associated with invertebrates or plants. These bacteria often display a remarkable metabolic flexibility, allowing them to adapt to very different environments in terms of nutrient availability (both quantitatively and qualitatively). In addition, they can display a repertoire of stress response mechanisms, which enables them to withstand even drastic changes in physicochemical conditions such as pH, temperature and oxygen availability. Changes in metabolic pathways, as well as induction of stress responses, very often affect biofilm formation and cell motility. Indeed, although motile bacteria can react to stress conditions via negative chemotaxis (Sampedro *et al.* 2015; Johnson and Ottemann 2017), *i.e.*, by swimming away from a stressful environment, induction of stress responses usually results in the transition from single cells to biofilm and in loss of flagellar motility (Crespo *et al.* 2017; Feng *et al.* 2018). Interestingly, induction of negative chemotaxis can itself be a trigger for biofilm formation and for other mechanisms of adaptation to unfavourable conditions (He and Bauer 2014). In fact, biofilm formation can itself be considered as a general stress response (reviewed in Landini (2009)) involving a variety of regulatory mechanisms in different bacteria. In Gram negative bacteria, the signal molecule c-di-GMP plays an important role in this process by inhibiting flagellar motility, while promoting production of adhesion factors and EPS through regulation of either gene expression or protein activity (Pesavento *et al.* 2008; Boehm *et al.* 2010; Paul *et al.* 2010; Hengge *et al.* 2016; Sadiq *et al.* 2017).

In this review, we will focus on three examples of motile Gram negative bacteria (*Escherichia coli*, *Pseudomonas*

aeruginosa and *Burkholderia* species) that can be found both in humans, where they can cause disease, and in the environment. In particular, we will discuss how host-related signals and environmental cues can impact production of different adhesion factors in *E. coli*, and how they trigger the activity of different, but interconnected, regulatory networks in *P. aeruginosa*. Finally, we will outline how flagellar motility relates to pathogenicity in *Burkholderia*. Our aim is to highlight examples of environmental cues affecting the balance between the planktonic and biofilm modes in these Gram negative bacteria, and how this process is relayed to virulence and host adaptation mechanisms.

Suboptimal environmental conditions positively affect adhesion factor production in *E. coli*

Curli fibres and cellulose as environmental biofilm determinants

Among the large repertoire of adhesion factors, both proteinaceous and EPS, possessed by *E. coli*, curli fibres (also known as thin aggregative fimbriae in *Salmonella*) are probably the most widely conserved. Curli fibres were originally described as a binding factor to fibronectin (Hammar *et al.* 1995), a family of eukaryotic glycoproteins, thus suggesting a role in adhesion to host cells. This hypothesis was also supported by subsequent observations that curli fibres can enhance bacterial internalization into human epithelial cells (Gophna *et al.* 2001). However, curli fibres do not bind specifically to fibronectin, and they can promote attachment to any abiotic surface, as well as cell aggregation (Prigent-Combaret *et al.* 2000). In most enterobacteria, curli fibres production is triggered by a combination of suboptimal growth conditions and/or environmental stresses, such as slow growth, nutrient starvation, low temperature (below 32°C), and low osmolarity (Römling *et al.* 2000). The master regulator relaying environmental stresses to expression of the curli-encoding *csg* genes is the general stress factor σ^S, itself induced by slow growth and nutritional stresses. Altogether, conditions allowing curli production are found outside of the intestinal tract of warm blooded animals, suggesting that curli might be synthesized when *E. coli* cells are shed from a host into the external environment. As host-related conditions tend to downregulate curli production, their involvement in bacteria–host interaction and colonization appears unlikely. Production of curli fibres is co-regulated with cellulose via the regulatory protein CsgD, which controls the expression of the *csgBAC* operon, encoding the structural components of curli, and the *dgcC* (formerly *adrA*) gene (Zogaj *et al.* 2001). In turn, the product of the *dgcC* gene stimulates the activity of the bacterial cellulose synthase (Bcs) complex by synthesizing c-di-GMP, which acts as an allosteric effect of cellulose synthesis (Römling *et al.* 2005) (Fig. 1). It might be hypothesized that, when *E. coli* is shed into the external environment from a warm blooded host, the CsgD protein activates a coordinated response leading to attachment to the surface and production of cellulose, in order to withstand environmental stresses like desiccation. CsgD-dependent regulation of curli and cellulose involves a complex network ultimately depending on σ^S: although initially thought to bind both the *csgDEFG* and *csgBAC* promoters directly, via interaction with the Crl protein (Pratt and Silhavy 1998), σ^S more likely controls *csgD* expression indirectly, via activation of yet another regulatory gene, *mlrA*, and of a c-di-GMP trigger enzyme network which controls MlrA activity by sequestering the protein (Lindenberg *et al.* 2013).

Regulation of curli gene expression is extremely complex (Gerstel and Römling 2003), involving at least 10 different transcription factors that can modulate the activity of the *csgD* promoter (Simm *et al.* 2014). The product of the *csgD* gene is in turn a regulatory protein able to activate the *csgBAC* operon, encoding the curli functional subunits (Römling *et al.* 2000), but also additional genes, mostly involved in production of extracellular factors and in the adaptation to the biofilm lifestyle (Zogaj *et al.* 2001; White *et al.* 2003; Gualdi *et al.* 2007). More recent works have focused on the role of regulatory RNAs on curli production, showing how the untranslated region of the *csgDEFG* operon is a hub for small RNA-dependent regulation, with up to seven RNAs (OmrA, OmrB, RprA, McaS, GcvB, RydC and RybB) binding this target region (Holmqvist *et al.* 2010; Jørgensen *et al.* 2012; Mika *et al.* 2012; Thomason *et al.* 2012; Serra *et al.* 2016). The reason for such a redundancy in regulators is not forthright, but it might reflect the convergence of a large number of environmental signals (*e.g.*, osmolarity, nutrient starvation, etc.) into a single response, namely, curli-mediated biofilm formation. However, while in some cases the mechanism relaying an environmental or physiological cue to curli gene expression is relatively straightforward, *e.g.*, slow growth rate via σ^S and Crl (Pratt and Silhavy 1998; Römling *et al.* 2000) or osmolarity and OmpR (Vidal *et al.* 1998), in some cases such a direct relationship remains more elusive, such as for temperature sensing. Indeed, while it was initially proposed that Crl-dependent regulation of the *csgBAC* promoter could be instrumental for only allowing efficient curli production at temperature not higher than 30°C (Bougdour *et al.* 2004), it appears that small RNA-dependent regulation (Holmqvist *et al.* 2010; Jørgensen *et al.* 2012; Mika *et al.* 2012; Thomason *et al.* 2012; Serra *et al.* 2016) and intracellular levels of the signal molecule c-di-GMP

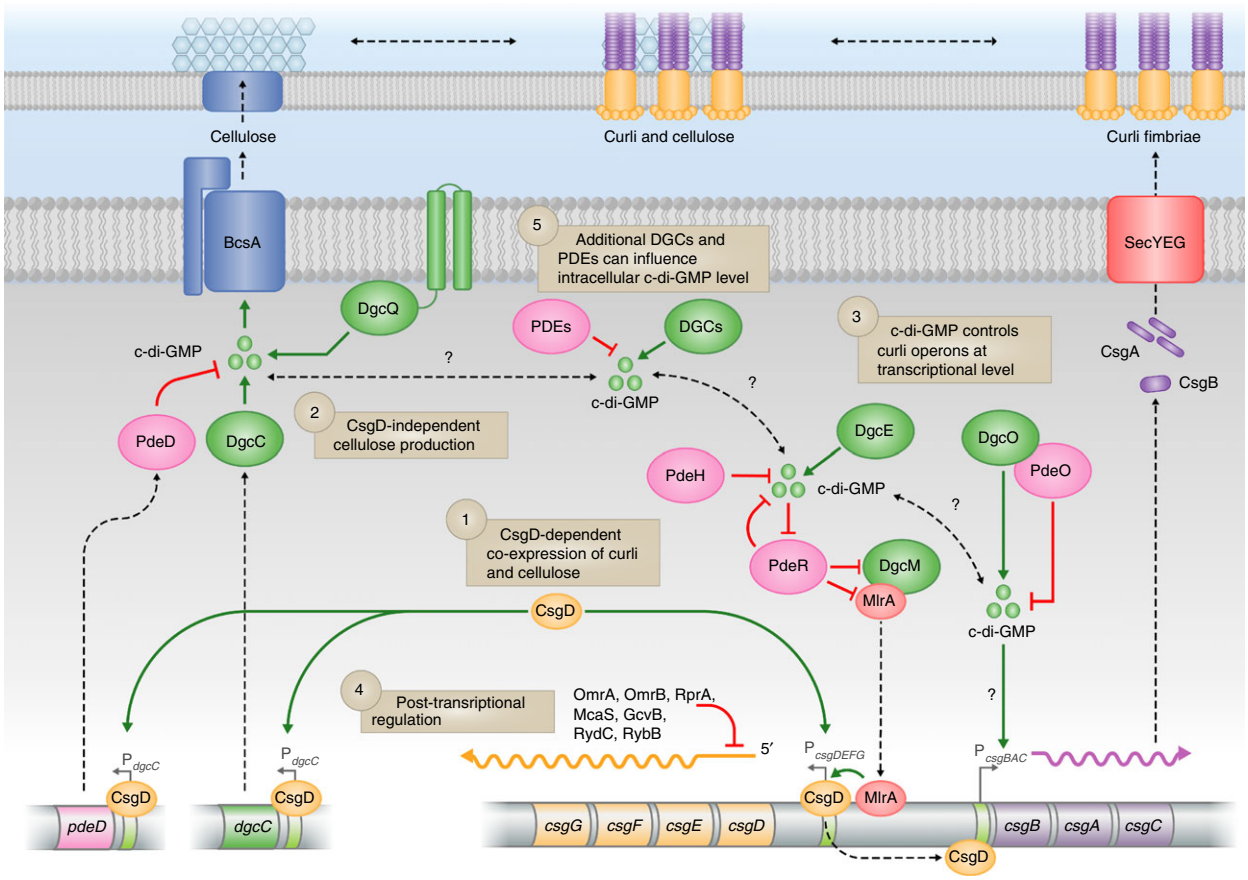

Figure 1 Schematic representation of the mechanisms controlling curli fibres and cellulose production in *Escherichia coli*. Curli and cellulose are co-expressed through the activity of the transcritpion factor CsgD (1), but production of the two structures can be uncoupled through the activity of the diganylate cyclases DgcQ (2). Expression of curli *csg* operons are controlled at transcriptional level by intracellular level of the second messenger c-di-GMP and a complex cascade of DGCs and phosphodiesterases (PDEs) that ultimately controls the transcriptional regulator MlrA (3, 5); in addition, the *csgDEFG* operon is regulated at post-transcriptional level by seven small RNAs (4). Solid lines indicate regulatory effects, positive (arrows) and negative (flat cap). Dashed lines can indicate either regulatory networks or directionality of a process (extracellular structure synthesis). Question marks indicate unkown/indirect molecular mechanisms. [Colour figure can be viewed at wileyonlinelibrary.com]

(Sommerfeldt *et al.* 2009), in addition to increased σ^S amounts in response to slower growth at low temperature (Landini *et al.* 2014), might be more directly involved in temperature-dependent regulation.

Although temperature regulation of curli is the most commonly found situation in enterobacteria, it is lost in some pathogenic *E. coli* strains, in which curli are also produced at host temperature (Römling *et al.* 1998). However, most pathogenic enterobacteria either display temperature-regulation of curli or are characterized by partial or total loss of the curli genes. For instance, curli-encoding genes are disrupted by deletions or insertions in a wide range of both *Shigella* and enteroinvasive *E. coli*, indicating that the pathogenic lifestyle might exert a strong selective pressure against curli production (Sakellaris *et al.* 2000). This selective pressure is probably due to the fact that curli amyloids, even if poorly expressed at host temperature, are highly immunogenic and even promote binding of antimicrobial peptides to the bacterial cells (Kai-Larsen *et al.* 2010). These results strongly suggest that the production of a cellular structure potentially important for virulence (as curli fibres can promote fibronectin binding and cell invasion) might actually be counterproductive for pathogenic bacteria, leading to its loss as part of an adaptation to the pathogenic lifestyle, and thus relegating curli fibres to environmental niches outside of the mammalian host.

Like curli fibres, also cellulose is produced in response to low growth temperatures, both in *E. coli* and in *Salmonella* species (Römling *et al.* 2003; Gualdi *et al.* 2008). This is hardly surprising, as cellulose and curli production are co-regulated, in a CsgD- and c-di-GMP-dependent fashion. Intracellular c-di-GMP concentrations are affected by several enzymes: diguanylate cyclases (DGCs)

synthesize it from two GTP molecules, c-di-GMP phosphodiesterases (PDEs) break it down either into a linear dinucleotide or in two GMP residues, and c-di-GMP trigger enzymes, which couple c-di-GMP hydrolysing activity to targeted protein-protein or protein-DNA interactions, thus regulating protein activity and/or gene expression. For extensive reviews on c-di-GMP signalling, we refer the readers to some of the excellent works that have been published over the last years (Römling *et al.* 2013; Hengge 2016). Co-regulation of curli and cellulose represents a paradigm of c-di-GMP regulation: expression of the *csgDEFG* operon responds to intracellular c-di-GMP concentrations and can be increased by the activity of different DGCs (Fig. 1) (Kader *et al.* 2006; Weber *et al.* 2006; Tagliabue *et al.* 2010). In turn, the CsgD protein, in addition to activating curli gene expression, also controls the *dgcC* gene, encoding a DGC able to activate the Bcs machinery (Zogaj *et al.* 2001). As the *csgDEFG* operon is only transcribed at growth temperatures lower than *c.* 32°C, lack of cellulose production at 37°C appears to be a consequence of *csgDEFG* downregulation. However, cellulose is produced at 30°C, but not at 37°C, in the *E. coli* laboratory strain MG1655 even when the CsgD protein is constitutively expressed (Gualdi *et al.* 2008), suggesting additional mechanisms of temperature control.

While simultaneous production of curli and cellulose is likely functional to strengthening the biofilm matrix, thus explaining its co-regulation, it has been shown that cellulose production can also be uncoupled from curli and controlled via a CsgD-independent mechanism. This CsgD-independent regulatory pathway also relies on c-di-GMP-dependent activation of the Bcs complex by a specific DGC, DgcQ, which allows cellulose production also at 37°C (Da Re and Ghigo 2006). Work performed in our laboratory suggests that the DgcQ protein can enhance cellulose production in response to intracellular concentrations of UTP, which triggers its activity, and of N-carbamoyl-aspartate, an intermediate of the pyrimidine *de novo* biosynthetic pathway, which in contrast inhibits the protein (Rossi *et al.* 2017). This mechanism of regulation would maximize DgcQ activity, and thus cellulose production, when *E. coli* cells are producing UTP from exogenous pyrimidine sources, rather than via the *de novo* pathway. This situation might take place, for instance, when *E. coli* is in the host gastro-intestinal tract, in which pyrimidines can reach millimolar concentrations (Vogel-Scheel *et al.* 2010). High exogenous pyrimidine concentrations would thus be sensed by the bacterium as a host-associated signal, triggering CsgD-independent, curli-uncoupled cellulose production as a host adaptation mechanism. Similar mechanisms, have been described in *Salmonella* Typhimurium in which cellulose production is coupled to arginine sensing (Mills *et al.* 2015), and in the *E. coli* probiotic strain Nissle 1917, in which cellulose production at 37°C, despite being dependent on c-di-GMP, does not require either CsgD/DgcC or DgcQ, thus suggesting that yet additional DGCs might be capable of affecting Bcs activity (Monteiro *et al.* 2009). These observations suggest that in the host environment, cellulose would mainly be produced independently of curli, promoting bacterial aggregation and adhesion to host cells, and modulating the host immune response (Monteiro *et al.* 2009; Ellermann *et al.* 2015; Tomić *et al.* 2016). Thus, adaptation to the warm blooded host might involve either through obliteration of production of extracellular structures such as curli and cellulose, more typical of pathogenic *E. coli* and *Shigella*, or via production of cellulose alone at host temperature, as mostly seen in several commensal and probiotic *E. coli* strains.

Flagellar motility *vs* biofilm in response to environmental signals

As already mentioned above, flagellar production and motility are downregulated in biofilm cells. A pivotal role in this process is played by c-di-GMP, which, in addition to upregulating production of adhesion factors, can negatively affect flagellar motility, both directly (Boehm *et al.* 2010) and through cellulose production (Zorraquino *et al.* 2013). However, motile and biofilm cells are not always mutually exclusive and can also contribute to the same cellular processes: indeed, flagella have been one of the first biofilm determinants to be identified, and they seem to play a role in early colonization of surfaces by bacterial cells (Pratt and Silhavy 1998). More recent observations point to a role of flagella also in mature biofilms (Serra *et al.* 2013). The interplay between flagellar motility and the biofilm state might be even more subtle in some pathogenic *E. coli*, in which biofilm structures can undergo quick dispersion and revert to a single cell state, possibly as a pathogenic strategy (Sheikh *et al.* 2002). Flagellar motility might allow quicker spreading of bacteria in the host, and contribute to initial binding and colonization of target cells: indeed, flagella are necessary for invasion of epithelial cells by pathogenic strains, such as adherent-invasive *E. coli* (AIEC; Barnich *et al.* 2003).

In some instances, flagella and adhesion factors can even be co-regulated. Indeed, the quorum sensing (QS)-related signal autoinducer 2 (AI-2) can activate both biofilm-related (Sperandio *et al.* 2001; Ren *et al.* 2004; González Barrios *et al.* 2006; Herzberg *et al.* 2006; Li *et al.* 2007) and flagellar and chemotaxis genes (Domka *et al.* 2006), through complex, and not yet fully elucidated, mechanisms which seem to involve the class I regulator of the flagellar genes, FlhDC, and the MqsA/MqsR

toxin-antitoxin system (Yamaguchi *et al.* 2009; Pesavento and Hengge 2012).

While the precise nature of the signals controlling AI-2-dependent regulation of extracellular structures remains elusive, another environmental signal, namely, the availability of exogenous glucose, also has a major effect on both adhesion factors and flagella expression. In *E. coli*, the main regulatory mechanism linking glucose sensing to gene expression is represented by the cAMP/CRP system (reviewed in Kolb *et al.* (1993)). In the absence of exogenous glucose, cAMP is synthesized and can bind to the transcription regulator CRP. Genes activated by CRP are therefore downregulated when glucose is present. Both curli and flagellar genes are regulated, either directly (curli) or via regulation of the *flhDC* operon (flagella) (Zheng *et al.* 2004). In addition, curli gene expression is also activated by the glycolytic flux regulator Cra (Reshamwala and Noronha 2011), whose molecular effector, namely, the glycolytic intermediate 1,6-fructose bisphosphate, prevents binding of the Cra protein to its target promoters (Ramseier 1996), thus strengthening the negative effect of glucose on curli production. Since high concentrations of glucose are present almost exclusively in the host environment (e.g. 0·7–1·2 g l^{-1} are typical concentrations in the blood), it is possible to speculate that extracellular structures might be downregulated in the presence of glucose in order to reduce their antigenic potential. Interestingly, flagellar expression is not affected by glucose availability in AIEC (Migliore *et al.* 2018), in which flagellar motility is needed for adhesion to epithelial cells (Barnich *et al.* 2003), suggesting that loss of glucose regulation might be a possible pathoadaptive mutation in this strain.

Regulatory networks connecting environmental signals to biofilm formation in *Pseudomonas aeruginosa*

Pseudomonas aeruginosa biofilm formation is linked to sensing of host environment

Pseudomonas aeruginosa is considered an environmental opportunistic pathogen able to infect a wide range of hosts (Rahme *et al.* 2000) and to cause a variety of different infections in humans, including urinary tract infections, otitis media, bacterial prostatitis and several chronic respiratory pathologies, such as cystic fibrosis (CF), primary ciliary dyskinesia and chronic obstructive pulmonary disease (Bjarnsholt *et al.* 2009; Sommer *et al.* 2016). In this bacterium, the switch from motile to sessile cells is pivotal for its ability to colonize the host: indeed, motility is required for the initial acute infection, in which a large set of virulence factors is produced, often followed by the development of a chronic infection, characterized by the loss of most virulence traits accompanied by the transition to the biofilm lifestyle (Smith *et al.* 2006; Gellatly and Hancock 2013). It is not uncommon, however, to consider biofilm formation itself as a *bona fide* virulence trait, considering that it allows *P. aeruginosa* to persist in the host environment despite the activity of immune system and antibiotics, often leading to incurable chronic infections (Hoyle and Costerton 1991; Jensen *et al.* 2010; Mulcahy *et al.* 2014).

In *P. aeruginosa,* initial attachment to surfaces takes place via the polar flagellum, followed by block of flagellar rotation and production of proteinaceous adhesins type IV pili, which secure bacteria to the surface and allow cells to spread over the surrounding area. Clonal growth produces microcolonies that, through accumulation of extracellular substances, including the exopolysaccharide Pel, Psl and alginate, as well as extracellular DNA, develop into a mature three-dimensional structure (O'Toole and Kolter 1998; Klausen *et al.* 2003a,b; Barken *et al.* 2008; Conrad *et al.* 2011; Bruzaud *et al.* 2015). Additional extracellular appendages such as Cup fimbriae, and the outer membrane adhesin CdrA, contribute to cell adhesion and biofilm structural integrity (Vallet *et al.* 2001; Kulasekara *et al.* 2005; Borlee *et al.* 2010). At the end of their life cycle, biofilms can actively disassemble, spreading motile and virulent cells in the surrounding environment (Karatan and Watnick 2009).

Different extracellular structures are involved at different steps and can play similar roles during biofilm formation: flagella are not always necessary for the initial adhesion (Klausen *et al.* 2003b) and contribute to the consolidation of a mature macro colony cap together with type IV pili (Barken *et al.* 2008); similarly, the Pel polysaccharide can partially substitute for the role of type IV pili during formation of micro colonies in a nonpiliated strain (Vasseur *et al.* 2005), but it can also work as a scaffold that maintains the community structure intact (Colvin *et al.* 2011). Finally, exopolysaccharides can increase tolerance to antibiotics, immune system and other stresses, by working as physical and chemical barriers (Wei and Ma 2013). The partial overlap in the function of extracellular structures suggests that, although the relative composition of the biofilm matrix can vary depending on the external conditions, and thus on the specific niche colonized by the bacterium, the biofilm development process remains unaltered (Monds and O'Toole 2009). However, preferential utilization of a given adhesion factor rather than another is also strongly affected by genetic variation in different *P. aeruginosa* strains.

It can thus be fair to say that, in each *P. aeruginosa* community, different cues determine the balance between

motile and sessile forms and, if transition to biofilm takes place, which determinants are produced. This decision process is the results of an extremely intricate and tightly interconnected network which relays on several regulatory mechanisms. Quorum sensing is possibly the best characterized regulatory network in *P. aeruginosa* and QS-dependent regulation has been extensively reviewed (Papenfort and Bassler 2016). In addition to QS, several regulatory pathways have been shown to impact biofilm formation and the switch between motile/sessile cells. Here, we will focus on three additional regulatory mechanisms and cues that can modulate their activity, namely: (i) cAMP/Vfr and glucose sensing; (ii) c-di-GMP and ppGpp; (iii) RNA thermometers and temperature sensing. The cAMP/Vfr and c-di-GMP-controlled regulatory networks are summarized in Fig. 2.

Regulatory networks involved in biofilm and motility of *P. aeruginosa*

cAMP/Vfr and glucose sensing

The second messenger cAMP and its response regulator Vfr control expression of multiple virulence factors involved in the phase of acute infection. Cyclic-AMP is synthesized from an ATP molecule through the activity of adenylate cyclases (ACs). Of the three ACs encoded in *P. aeruginosa* PAO1 genome, only CyaA and CyaB contribute to modulating the intracellular cAMP level (Stevens *et al.* 2014). In laboratory conditions, the majority of cAMP is synthesized through the activity of the CyaB enzyme (Wolfgang *et al.* 2003). Activity of CyaB is increased upon surface contact, a stimulus relayed through the concerted action of type IV pili and the mechanochemical sensor system Chp (Persat *et al.* 2015). At physiological pH, CyaB is also regulated in response to CO_2/HCO_3^- (Topal *et al.* 2012), whose levels in eukaryotic hosts are relatively high. Thus, it is reasonable to believe that CyaB might integrate both mechanical and chemical signals specifically connected with the host environment and regulate cAMP levels accordingly. Cyclic-AMP acts as coactivator for the Vfr transcriptional regulator: the cAMP/Vfr complex promotes the expression of virulence factors involved in the acute phase of *P. aeruginosa* infections, including type III secretion systems and type IV pili (Wolfgang *et al.* 2003). At the same time, cAMP/Vfr represses the expression of FleQ (Bucior *et al.* 2012), thus inhibiting flagellar motility, adhesion and biofilm formation on abiotic surfaces (Ono *et al.* 2014).

Although AC activity in *P. aeruginosa* is not strictly dependent on glucose sensing, cAMP signalling impairs adhesion in response to glucose availability: indeed, glucose-starvation promotes biofilm dispersion via a partially characterized mechanism that requires the activity of CyaA, the cytosolic AC, but not the Vfr regulator (Huynh *et al.* 2012). As already discussed in the context of flagellar regulation in *E. coli*, high glucose concentrations (in the g l^{-1} range) can only be found in the host, particularly in the blood and in the interstitial liquids of animals; upon induction of strong inflammatory processes, glucose concentrations can increase in various tissues, such as the airway epithelium of CF patients (Garnett *et al.* 2013). Thus, increase in glucose availability can

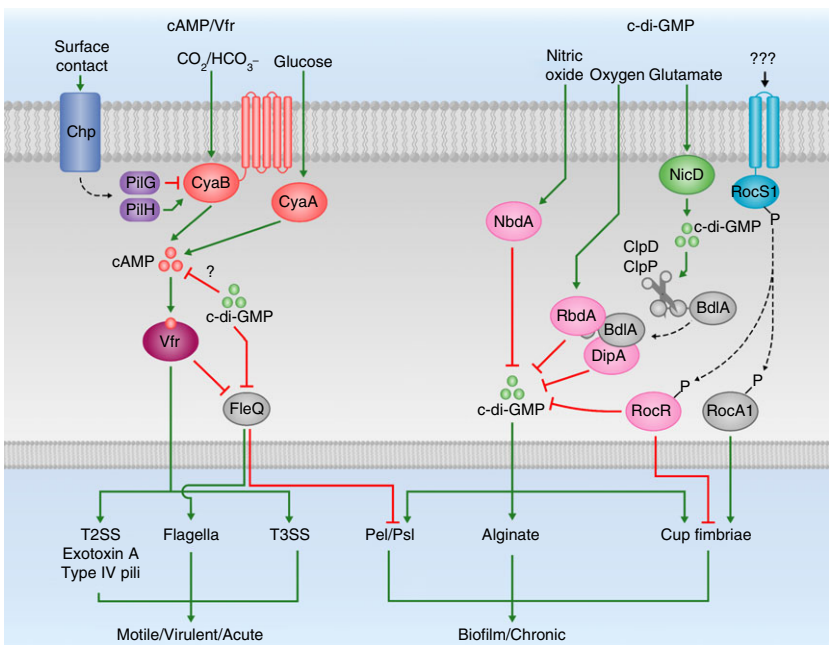

Figure 2 Represenativie environmental and host-related cues modulating cAMP/Vfr and c-di-GMP signalling networks and their effect on *Pseudomonas aeruginosa* physiology. Solid lines indicate regulatory effects, positive (arrows) and negative (flat cap). Dashed lines can indicate either regulatory networks or directionality of a process (phosphate transfer in two components regulatory systems). Question marks indicate unkown/indirect molecular mechanisms or signals. [Colour figure can be viewed at wileyonlinelibrary.com]

provide a cue to induce a preventive response to the host immune system in this bacterium. Unlike *E. coli*, in *P. aeruginosa* glucose does not represent the favoured source of carbon (Palmer *et al.* 2005). It is, however, actively transported into the cell and consumed upon oxidation to gluconate. Interestingly, the gluconate-responsive regulator PtxS, which controls gluconate uptake and degradation, can interact with the transcriptional regulator PtxR (Daddaoua *et al.* 2012) directly controlling the expression of *P. aeruginosa* toxin ToxA, as well as of LasB (Hamood *et al.* 1996) and indirectly, of Cup fimbriae (Qaisar *et al.* 2016). PtxR is directly regulated by cAMP/Vfr (Ferrell *et al.* 2008): thus, PtxS–PtxR interaction could bridge cAMP/Vfr signalling and glucose sensing, to trigger host adaptation mechanisms, including production of virulence factors. Finally, the PtxS–PtxR system might link glucose and temperature sensing, as described in the 'Role of temperature sensing in *P. aeruginosa* biofilm formation' section below.

Another regulatory system connecting sugar metabolism to virulence and biofilm formation is the CbrA/CbrB two component regulatory system (Abdou *et al.* 2011; Yeung *et al.* 2014). Indeed, mutations in the *cbrA* and *cbrB* genes affect the utilization of various sugars by *P. aeruginosa*, but also impair virulence, while promoting biofilm formation, again suggesting that pathogenesis is more associated with motile cells, and providing another example of a link between glucose sensing and activation of virulence determinants.

Through the effects of various signalling mechanisms, glucose availability strongly impacts biofilm architecture: cells grown in the presence of glucose develop mushroom-shaped macro colony, specifically requiring flagella and Tfp for initial adhesion, which are in contrast dispensable when *P. aeruginosa* is grown on citrate, lactate or amino acids (Klausen *et al.* 2003b). These compounds are found at high levels in host fluids such as wound exudates, urine and CF mucus (Trengove *et al.* 1996; Brooks and Keevil 1997; Palmer *et al.* 2007), and might possibly contribute to adaptation to host niches where glucose concentrations are low. At any rate, production of different biofilm determinants in response to different carbon sources represents an example of how primary metabolism can strongly affect cellular processes such as adhesion and biofilm formation.

c-di-GMP

While high levels of cAMP promote acute virulence and inhibit biofilm formation (Wolfgang *et al.* 2003), increasing concentrations of the second messenger c-di-GMP promote sessility (reviewed in Valentini and Filloux (2016)), in line with the general role of this molecule in bacteria. *Pseudomonas aeruginosa* genome carries one of the highest number of DGC- and PDE-encoding genes among Gram negative bacteria (Valentini and Filloux 2016). Production of alginate, arguably the most abundant EPS in *P. aeruginosa*, is activated in response to c-di-GMP synthesized by the membrane-bound MucR DGC (Hay *et al.* 2009) via the Alg44 protein (Merighi *et al.* 2007; Oglesby *et al.* 2008). Similarly, c-di-GMP modulates production of Pel polysaccharide both at the synthesis level through the PelD protein (Lee *et al.* 2007; Whitney *et al.* 2012) and at expression level acting on the *pel* operon through the FleQ transcriptional regulator. At low c-di-GMP concentrations, FleQ is an activator of flagella biosynthesis and a repressor of *pel* operon. Upon c-di-GMP binding, FleQ dissociates from the protein FleN relieving its negative effect on *pel* transcription and stimulating Pel synthesis (Hickman and Harwood 2008; Baraquet *et al.* 2012; Matsuyama *et al.* 2016). In addition, FleQ is responsible for modulating, in a c-di-GMP-dependent manner, the expression of genes involved in Psl exopolysaccharide production and the adhesin CdrA (Baraquet and Harwood 2015). Finally, Cup fimbriae-encoding *cupB* and *cupC* genes are regulated by the Roc-SAR signalling system, which relays on the activity of yet another PDE, RocR, for modulating adhesion and biofilm formation (Kulasekara *et al.* 2005). While c-di-GMP promotes sessility and biofilm formation, ppGpp, the other main signal molecule derived from GTP, seems to have the opposite effect, increasing *P. aeruginosa* pathogenesis (Xu *et al.* 2016). *Pseudomonas aeruginosa* mutants unable to produce ppGpp are avirulent in several animal infection models (Pletzer *et al.* 2017), although it is not yet clear whether lack of pathogenesis results from impaired expression of virulence factors or to inability to adapt to host niches often limiting for various amino acids. Indeed, auxotrophic mutants for amino acids are also avirulent and have been proposed as possible live vectors for antigen delivery (Epaulard *et al.* 2008).

Although it is clear that the temporal accumulation of c-di-GMP promotes biofilm development while inhibiting virulence and motility, only a small number of environmental signals have been described as directly acting on DGCs and PDEs. For instance, upon glutamate sensing, the DGC NicD is activated leading to a transient increase in c-di-GMP levels (Basu Roy and Sauer 2014), which mediates an unusual nonprocessive ClpP/ClpD-mediated proteolysis of the BdlA regulator (Petrova and Sauer 2012a). BdlA recruits and activates the PDEs DipA and RbdA, reducing the overall intracellular concentration of c-di-GMP and promoting biofilm dispersal (Petrova and Sauer 2012a,b). Interestingly, RbdA also contains a conserved PAS domain responsible for modulating PDE activity in response to low-oxygen (An *et al.* 2010), thus

integrating unrelated cues within the same signalling cascade.

Nitric oxide (NO) is another strong signal that impacts biofilm formation mediating its dispersal through c-di-GMP signalling in different bacteria, including *P. aeruginosa* (Barraud *et al.* 2009; Cutruzzolà and Frankenberg-Dinkel 2016). NO can derive either from denitrification, a common metabolic process in environmental *Pseudomonas* species growing in oxygen-limiting environments such as anoxic waters and soils; in *P. aeruginosa*, however, NO-sensing is strongly linked to the activity of the host immune system and by the generation of nitrogen reactive species in lysosomes (Kolpen *et al.* 2014). Although potentially relying on the activity of multiple sensors (Cutruzzolà and Frankenberg-Dinkel 2016), NO-mediated biofilm dispersal requires the activity of the NbdA PDE, that senses the level of NO through the conserved MHYT domain (Li *et al.* 2013).

Role of temperature sensing in *P. aeruginosa* biofilm formation

In the *P. aeruginosa* PA14 strain, growth at host temperature (37°C) results in the differential expression of *c.* 7% of total genes in comparison to growth at 28°C, *i.e.*, a temperature more representative of the external environment (Wurtzel *et al.* 2012). Among the virulence factors induced at host temperature, genes encoding type III secretion systems and the phenazine biosynthetic pathway, necessary for the production of the toxic compound pyocyanin, showed strong temperature-dependent regulation. In addition, the same study shows that the anti-sigma factor MucA is also induced, slightly but significantly, at host temperature (Wurtzel *et al.* 2012). By sequestering the alternative sigma factor AlgU/T (Schurr *et al.* 1996), MucA downregulates genes involved in alginate production (Wei and Ma 2013). Mutations in the *mucA* gene lead to alginate hyperproduction, mucoidity and are often found in *P. aeruginosa* isolates from CF patients; thus, an even slight increase in *mucA* expression in response to the host temperature might tip the scale towards the motile/virulent phenotype rather than the biofilm/sessile mode.

Temperature-dependent gene expression regulation can take place via different mechanisms, including changes in the nucleic acids secondary structure; one example of such mechanisms are RNA thermometers (RNATs), thermolabile secondary structures that regulate translation in response to environmental temperature and are often connected with the control of virulence factors (Kortmann and Narberhaus 2012; Righetti *et al.* 2016). RNATs have been found in the 5′-UTR of the *ptxS* gene (Delvillani *et al.* 2014). PtxS interacts in a gluconate-depended manner with the PtxR transcriptional regulator (Daddaoua *et al.* 2012) controlling expression of the ToxA toxin (Hamood *et al.* 1996) and indrectly of Cup fimbriae (Qaisar *et al.* 2016), as already discussed in the '*cAMP/Vfr and glucose sensing*' sections. Thus, regulation of *ptxS* through an RNA thermometer and of its interaction with PtxR in response to gluconate, *i.e.*, of a glucose metabolite, could activate production of Toxin A only at 37°C and when glucose is available (Delvillani *et al.* 2014), conditions that can only be found in the warm blooded host.

Role of motility and biofilm determinants in pathogenicity of *Burkholderia* species

The *Burkholderia* genus: an outlook

In 1992, seven species belonging to the *Pseudomonas* genus were reassigned to a new genus, named *Burkholderia* (Yabuuchi *et al.* 1992), based on the following characteristics: aerobic Gram negative rods, catalase and oxidase positive, able to accumulate poly-hydroxy-butyrrate as carbon storage, mesophiles, and lacking ability to produce pigments. Of the seven species initially included in the *Burkholderia* genus, the only nonmotile one was *B. mallei*, the only obligate pathogen in the genus. Subsequent work has expanded the *Burkholderia* genus to around 120 species, which inhabit remarkably diverse ecological niches, ranging from soil and water to plant rhizosphere, insects, fungi, hospital environments and humans (Coenye and Vandamme 2003). This extreme flexibility correlates with the average size of the *Burkholderia* genome (6–7 Mbp) and with their ability to acquire novel genes by horizontal transfer. Typically, the genome is constituted by two chromosomes of different sizes, one encoding the housekeeping functions and the primary metabolism genes, while the second one carries mostly related to adaptation to different environments (Wigley and Burton 2000). A very extensive functional transcriptomics study performed on *B. pseudomallei* indicates that genes carried on Chromosome II are much more likely to be regulated in response to environmental signals (Ooi *et al.* 2013). *Burkholderia* species are often found in consortia, such as for the *Burkholderia cepacia* complex (BCC), comprising up to 20 species, including a few opportunistic pathogens, such as *B. dolosa*, *B. multivorans* and *B. cenocepacia*; BCC can infect immunocompromised subjects and cause an often fatal lung infection in individuals affected by CF (Mahenthiralingam *et al.* 2005). Like *P. aeruginosa*, BCC can infect a variety of different hosts, both animals and plants, and represents a typical case of 'environmental pathogen', perfectly adapted to the external environment but able to infect

various hosts. In the next section, we will focus on three related species *B. thailandensis*, *B. pseudomallei*, and *B. mallei*, respectively avirulent for the mammalian hosts, opportunistic pathogen able to cause the very severe disease melioidosis, and obligate pathogen causing glanders, mostly in equines, as an example of transition from environmental to pathogenic bacterium.

Production of EPS and other cell surface-associated factors in response to environmental signals in *B. pseudomallei*

Burkholderia pseudomallei, the etiological agent of melioidosis, a severe disease endemic in many parts of South East Asia, is an environmental bacterium found in soil and surface waters, especially in subtropical countries (Dance 2000). In the human host, *B. pseudomallei* is an intracellular pathogen, able to escape the endosome, to move within the host cell cytoplasm and spread to neighbouring cells using actin-based motility, to promote formation of multi-nucleated giant cells, and to evade host cell autophagy (reviewed in Allwood *et al.* (2011)). Thus, in this bacterium, sensing the transition from an environmental reservoir to the human host requires the ability to sense and respond to changing environmental cues, and the timely production of virulence factors. Unlike the BCC complex, where capsular EPS production is higher in strains more adapted to the human host, in *B. pseudomallei* it is mostly environmental isolates that display smooth and mucoid morphologies, while clinical isolates display rough, wrinkled and dry colonies (Chen *et al.* 2009), which might suggest that capsular production is more functional to the external environment than to the host. However, production of a capsular polysaccharide does play an important role in *B. pseudomallei* virulence (Reckseidler *et al.* 2001; Lazar Adler *et al.* 2009), although, once again, its role, rather than being an adhesion factor, might be to protect bacterial cells from the host immune response. This notion is supported by the observation that noncapsulated *B. pseudomallei* mutants can invade human cells *in vitro* even more efficiently than the wild type (Phewkliang *et al.* 2010). Likewise, strains of the closely related bacterium *B. thailandensis* that have inherited *B. pseudomallei* capsular genes by horizontal transfer are still avirulent in mammals, suggesting that additional factors are required for pathogenesis. In contrast, more virulent isolates of *B. thailandensis*, that have been reported to cause occasional infections in immunocompromised individuals, do not necessarily produce the *B. pseudomallei*-like capsule (Sim *et al.* 2010).

As it can be expected from its ability to adapt to very different environments, *B. pseudomallei* can form biofilm and produce adhesion factors in response to several environmental cues. Several reports suggest that biofilm formation in *B. pseudomallei* is a complex process and can be modulated in a strain-specific manner by environmental signals such as temperature, pH, and glucose content. In particular, it has been demonstrated that medium composition (e.g. high glucose, LB *vs* M9 medium) and low temperature (27–30°C compared to 37°C) provide ideal conditions for microcolony formation, enhance association with epithelial cells and promote biofilm formation in clinical strain of *B. pseudomallei* (Brown *et al.* 2002; Boddey *et al.* 2006; Ramli *et al.* 2012). These data suggest that some virulence factors promoting adherence to eukaryotic cells might have evolved in the original soil habitat of this bacterium, possibly as a mechanism to invade the original hosts for *B. pseudomallei*, such as amoebae or invertebrates, which, incidentally, are also successfully infected by *B. thailandensis* (Fisher *et al.* 2012). On the other hand, Kamjumphol *et al.* (2013) demonstrated that also high osmolarity (NaCl) and high amount of iron, in addition to low temperature, promote biofilm production while inhibiting protease production, swimming and swarming motility, *i.e.* virulence-related traits.

While in many environmental pathogens, temperature around 37°C constitute a host-related signal and induce host adaptation and/or production of virulence determinants (see previous sections), this pattern does not appear to be so clear cut in *B. pseudomallei* in which, as mentioned in the previous paragraph, some adhesion factors involved in epithelial cell invasion display an optimal expression at low temperature. Temperature changes also regulates flagellar production both in *B. pseudomallei* and in *B. thailandensis*: in the latter species, flagellar motility is strongly affected at 37 *vs* 28°C, due to negative regulation of the flagellar operon at the mRNA stability level at the host temperature (Peano *et al.* 2014). Negative regulation of flagellar expression at host temperature has also been reported in *Listeria monocytogenes*, another opportunistic pathogen (Kamp and Higgins 2011). Interestingly, mutations abolishing flagellar motility negatively affect pathogenesis in *B. pseudomallei* (Chua *et al.* 2003) and also in *B. cepacia* (Tomich *et al.* 2002), suggesting that the flagellum is a virulence factor in these bacteria. Temperature-dependent downregulation of flagellar genes has also been observed in *B. pseudomallei*, but it only has very little effects on cell motility (DeShazer *et al.* 1997; Ooi *et al.* 2013). These observations might suggest that lack of flagellar motility at 37°C in *B. thailandensis* can contribute to its being avirulent in the mammalian host. However, the picture is complicated by the fact that in *B. mallei*, the only obligate pathogen in the *Burkholderia* genus, flagellar genes are interrupted by insertion sequences, resulting in lack of motility, likely a

pathoadaptive mutation (Galyov *et al.* 2010). Thus, pathogenic *Burkholderia* species appear to have adopted very different strategies in terms of utilization of flagellar motility during host infection, as flagella provide an advantage in spreading the infection, but are also a highly antigenic structure.

Oxygen deprivation represents another important host-related signal for environmental pathogens and affects production of virulence factors in *Burkholderia* species. Anoxic conditions strongly stimulate production of EPS in *B. cenocepacia* (Pessi *et al.* 2013) and of LPS in *B. thailandensis* (Peano *et al.* 2014). The observation that growth conditions largely unfavourable for bacteria with a strong preference for aerobic respiration can trigger such an energy costing process strongly suggests that EPS production represents an important response to stress conditions and a possible defence mechanism from environmental predators and host immune response, in line with what already described for other bacteria. It is likely that long term adaptation to unfavourable environmental conditions are the ultimate drive to the emergence of more host-adapted, and often more pathogenic, mutant variants (Bragonzi *et al.* 2017). In the perspective of long-term adaptation to the host, it should be reminded that *B. mallei*, like many other bacterial pathogens, has taken a 'reductionist' approach, reducing its genome size which, although still rather large at its 5·8 Mbp, it is significantly smaller than *B. thailandensis* (6·7 Mbp) and *B. pseudomallei* (7·2 Mbp), and carries many inactivated or non-functional genes such as the flagellar operons (Nierman *et al.* 2004).

Conclusions

In this article, we have focused on how adhesion factors, biofilm determinants and cell motility are affected by environmental signals in different bacteria and have described examples of regulatory mechanisms that relay such signals to specific cell processes. We have focused on *E. coli*, *P. aeruginosa*, and the *Burkholderia* genus, namely, bacteria that share several basic aspects in their biology, being Gram negative rods, either aerobes or facultative anaerobes, capable of flagellar motility, prototrophs, and able to survive both in the external environment and in a variety of different hosts. Although they share some common features, even the few bacterial species we have considered display a remarkable variety and flexibility in the utilization of adhesion factors in response to environmental cues. For instance, cell motility is more related to pathogenesis, while biofilm formation and EPS production appear to be a defence mechanism towards the host immune response or environmental stresses. On the other hand, however, the obligate pathogen *B. mallei* is nonmotile. Likewise, production of cellulose is higher at temperature of 30°C and lower than at 37°C in most *E. coli* strains, despite the possible role of this EPS in cell adhesion and in protection from the host immune response, clearly indicating that in *E. coli* cellulose tends to be produced in response to environmental stresses such as desiccation. Finally, temperatures around 37°C are a major signal for induction of virulence factors in pathogenic bacteria like *P. aeruginosa*; yet, in *B. pseudomallei*, factors promoting adhesion to eukaryotic cells can be better expressed at much lower temperatures. Such extreme variety in the adaptation strategies to various environments, in the utilization of cell structure such as flagella and EPS, and in how environmental signals are relayed to cell processes should constitute a caveat when attributing to a given extracellular structure a specific role in processes such as pathogenesis, simply based on structure similarity or functional homology.

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Conflict of Interest

The authors declare no conflict of interest.

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