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New mechanistic insights into the motile-to-sessile switch in various bacteria with particular emphasis on *Bacillus subtilis* and *Pseudomonas aeruginosa*: a review

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ABSTRACT

A biofilm is a complex assemblage of microbial communities adhered to a biotic or an abiotic surface which is embedded within a self-produced matrix of extracellular polymeric substances. Many transcriptional regulators play a role in triggering a motile–sessile switch and in consequently producing the biofilm matrix. This review is aimed at highlighting the role of two nucleotide signaling molecules (c-di-GMP and c-di-AMP), toxin antitoxin modules and a novel transcriptional regulator BolA in biofilm formation in various bacteria. In addition, it highlights the common themes that have appeared in recent research regarding the key regulatory components and signal transduction pathways that help *Bacillus subtilis* and *Pseudomonas aeruginosa* to acquire the biofilm mode of life.

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Introduction

A unique facet of microbial life is the existence of a mode of life termed a biofilm, in which microbes become remarkably different from their planktonic form or sessile counterparts. A biofilm is considered to be the most enigmatic and abstruse feature of bacterial life. Biofilms or ‘cities of microbes’ (Hall-Stoodley & Stoodley 2009) are diverse microbial communities that grow in multicellular aggregates and burgeon on a variety of biotic or abiotic surfaces surrounding themselves with self-produced hydrated slimy secretions (Watnick & Kolter 2000), termed extracellular polymeric substances (EPS) or the ‘house of biofilm cells’ (Flemming et al. 2007). The plaque encrusting our teeth and gum margins, the green mats of algae on river surfaces, the gunge obstructing the water flow in pipes and the slippery surfaces on rocks in streams are some of the common paradigms of biofilms that can spread on any surface where there is moisture and nutrients. The understanding of bacterial biofilms has brought us to the realization that bacteria do not merely constitute a unicellular life but they can form highly organized communities with a bewildering array of complex interactions (Shapiro 1998).

Biofilm formation is an ancient and integral feature of many prokaryotes, that fossil records suggest were around

3.2 billion years ago (Hall-Stoodley et al. 2004). However, the first scientific evidence concerning biofilm formation came from Henrici when he was surprised to see that the bacteria in water formed a uniform coating on glass surfaces in association with fungal growth (O’Toole et al. 2000). He stated that ‘They are fairly firmly adherent to the glass, not removed by washing under a tap’ (Henrici 1933). It is now evident that biofilm formation is a common feature shared by most if not all bacteria, in natural, medical or engineered systems. Biofilms have received a great deal of attention especially during the last three decades, but a complete understanding of this ubiquitous mode of growth is elusive, because the understanding of this major mode of microbial life is slow and incremental. However, a plethora of recent research on biofilm formation under natural habitats or in laboratory controlled model systems have brought great revolutions in microbiology by elucidating many underlying mechanisms governing biofilm formation.

What happens that makes bacteria switch into another mode of life is an important mechanistic question that has been well addressed and reviewed by a number of researchers (O’Toole et al. 2000; Donlan & Costerton 2002; Jefferson 2004). Once bacteria in their planktonic form encounter a surface, they undergo a series

of dramatic changes at both the physiological and the genetic level. A full understanding of bacterial life and mechanisms involved in the transition from planktonic lifestyle to biofilm mode of life is critical to understand these complex microbial societies. A number of signal transduction systems have been identified which have a pivotal role in the transcriptional regulation of motility and biofilm formation. One class of signal transduction systems is the second messenger signaling system, which enables bacteria to respond to environmental changes and generate a second messenger signaling molecule in the cell. This diffusible signaling molecule binds directly to specific receptor proteins or RNA to alter their behavior (Lee 2016). It is well accepted that the second nucleotide signaling molecules play key roles in various cellular processes including biofilm formation. For instance, the intracellular second messenger molecule, 3',5'-cyclic diguanylic acid (c-di-GMP) plays a critical role in biofilm formation by controlling motility and EPS production through signal transduction, gene transcription, protein secretion and stability (Kalia et al. 2013). In recent years, another nucleotide signaling molecule (cyclic di-AMP) has appeared (Corrigan & Gründling 2013); however, its role in biofilm formation is not yet well explored, despite it being implicated in biofilm formation in many bacterial species. A great deal of attention has also been given to finding the role of toxin anti-toxin (TA) modules in biofilm formation. Several reports have described different ways in which these modules affect biofilm formation. However, there is no single report summarizing all possible ways in which these widely distributed small genetic modules affect biofilm formation.

The objectives of this article are to provide a summary of the current state of knowledge regarding the role of two nucleotide signaling molecules, cyclic-di-GMP and

c-di-AMP, various transcriptional regulators and toxin anti-toxin (TA) modules in mediating biofilm formation, and to outline future challenges. A detailed mechanism of biofilm formation in the most widely studied model of biofilm-forming Gram-positive (*Bacillus subtilis*) and Gram-negative (*Pseudomonas aeruginosa*) bacteria has been provided, to outline the role of all known transcriptional regulators and to summarize recent advances in understanding biofilm formation by these organisms.

Biofilm development

Biofilm formation is a complex dynamic process that involves coordination and communication among a multitude of bacterial species (Davey & O'Toole 2000). Bacteria have complex differentiation and communication systems, and it is now evident that biofilms also govern dynamic cellular differentiation throughout the development of the complex community (Vlamakis et al. 2008).

The development of biofilm usually has three major stages: (1) attachment of motile bacterial cells to a surface; (2) growth and proliferation of bacterial cells leading to the formation of a mature biofilm and subsequent production of EPS; (3) dispersal or detachment of bacterial cells (Allegrucci et al. 2006). Figure 1 shows three stages of biofilm formation by *Anoxybacillus flavithermus* strain B12 isolated from a milk powder. Figure 1a shows initial attachment where the grain boundaries of the stainless steel surface are clearly visible. In Figure 1b there is a greater coverage of bacterial cells, depicted by the grain boundaries being less visible due to the accumulation of organic materials and EPS. Once bacteria disperse from the surface they leave 'footprints' or depressions in the EPS representing the area that bacteria occupied before dispersal (Figure 1c). These figures show the formation of a

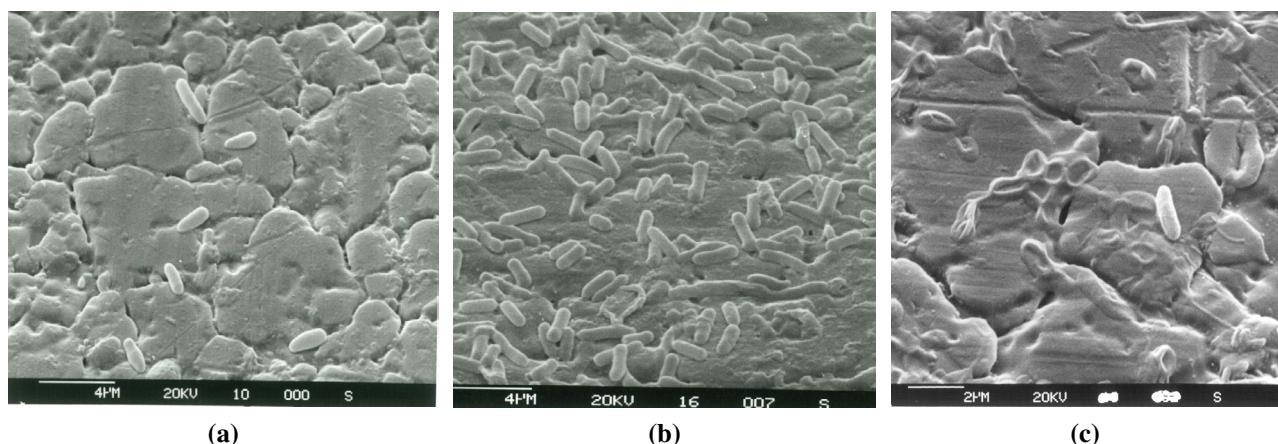


Figure 1. Three stages of monolayer biofilm formation on a SS surface by *A. flavithermus* strain B12 isolated from a milk powder sample. (a) Attachment of only a few cells and grain boundaries are clearly visible; (b) good coverage of cells on a SS surface with EPS production; (c) dispersal of single cells from the biofilm leaving "footprints" or depressions indicating the area that cells had previously occupied.

monolayer biofilm on stainless steel surface by *A. flavithermus*. It is known that most of the thermophilic bacilli form monolayer biofilms and hence lack the traditional form of a mature biofilm (Flint et al. 1997). However, strains of some thermophilic species can form multilayer biofilms and appear like a giant lump of cells at maturation. Figure 2 represents the final stage of a traditional biofilm formed by *Geobacillus stearothermophilus* on stainless steel (SS) after 24 h incubation in reconstituted skimmed milk.

Until recently, there has been a consensus on the biofilm formation model, based on the attachment of single cells on a surface leading to the development of a mature biofilm. However, Kragh et al. (2016) provided some novel insights into biofilm formation and suggested the revision of the existing rigorous model of biofilm development. It was proposed that at the first stage of biofilm formation, not only single cells in planktonic phenotypes but also multicellular aggregates can seed the surface. Single cells irreversibly attach to the surface while cell aggregates form a structure perpendicular to the surface and develop into an elevated structure which is much higher than that formed by the descendants of single cells surrounded by them. The mature biofilms shed single cells as well as cell aggregates.

Unlike in the planktonic lifestyle, bacteria shut off their motility in biofilms; however, some motile cells representing a minor proportion of the overall population can still be observed in a mature biofilm (Vlamakis et al. 2008). Sporulation within a biofilm is not an unusual process but it is not considered to be a requirement for biofilm formation (Branda et al. 2001). A mature biofilm would ultimately lead to the production of spores and highly motile vegetative cells. These vegetative cells are known as dispersal cells and are genotypic and phenotypic variants of the pioneer biofilm forming cells (McDougald et al. 2011). Thus, a mature biofilm comprises different types of sub-populations of bacterial cells which vary in gene expression profiles and physiological states (van Gestel et al. 2015; Besharova et al. 2016).

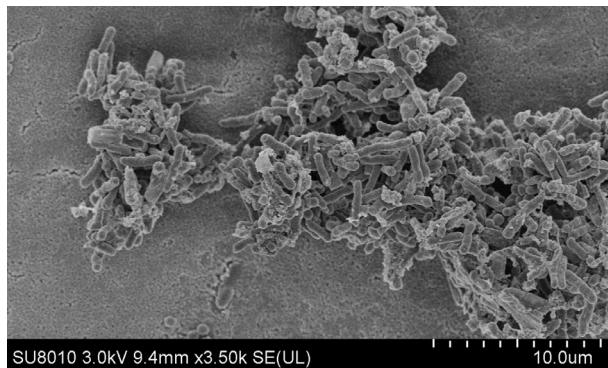


Figure 2. A multilayer biofilm of *G. stearothermophilus* on a SS surface in skimmed milk after 24 h.

Different mechanisms involved in the formation of biofilm include the redistribution of attached cells by surface motility (O'Toole & Kolter 1998), binary fission of already surface attached cells (Heydorn et al. 2000) and the incorporation of new cells (Tolker-Nielsen et al. 2000) and cellular aggregates from flowing liquid into the developing biofilms (Kragh et al. 2016). During this attachment process, EPS plays a pivotal role in mediating the transition from reversible to irreversible sticking of planktonic bacterial cells and also aids in further attachment of motile bacterial cells (Stoodley et al. 2002). Figure 3 shows a strong sticky framework of EPS surrounding and covering the cells of *G. thermoleovorans* in a 24-h biofilm on a SS surface.

EPS consists of extracellular DNA (e-DNA), proteins, water, cells, lipids, RNA, polysaccharides and various metabolites (Allison 2010; Flemming & Wingender 2010). A range of regulatory and enzymatic activities, as a result of interaction with the biofilm components, results in a dynamic and heterogeneous microenvironment required for the stability of architecturally complex biofilms (Allison 2010). Among the biofilm components, the role of e-DNA in biofilm formation and integrity has been acknowledged (see reviews by Das et al. 2013; Okshevsky & Meyer 2015).

Recent advances in chemical biology have revealed that all organisms, including minute eukaryotic organisms, possess a variety of regulatory mechanisms to smooth signal transduction in response to changing environmental cues (Ulrich et al. 2005). Bacteria communicate and coordinate through chemical signals by exuding a variety of compounds into their surroundings and may also generate some externalized structures that extend from their surfaces and are used in cell-to-cell communication (Visick & Fuqua 2005). Many cooperative

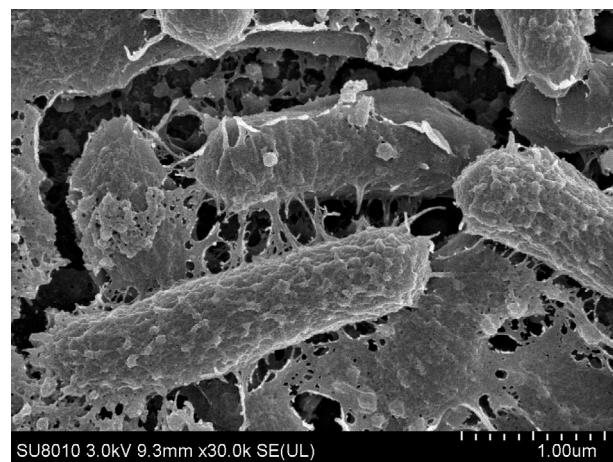


Figure 3. Visible EPS fibers covering the cells of *G. thermoleovorans* in a 24 h biofilm on a SS surface in skimmed milk.

activities and physiological processes in bacteria within biofilms are regulated by a mode of intracellular communication or a gene regulatory mechanism termed quorum sensing (Dobretsov et al. 2009; Li & Tian 2012) or cell-density sensing (Henke & Bassler 2004), during which bacterial cells release small diffusible extracellular signaling molecules (autoinducers) into the surrounding environment (Dunny & Leonard 1997; Miller & Bassler 2001; Senadheera & Cvitkovitch 2008). These chemical signaling molecules accumulate as a result of an increase in the bacterial population. Bacteria sense changes in their number or in the number of other bacterial species in their vicinity by monitoring fluctuations of the signal concentration, and they orchestrate population-wide global gene expression, leading to collective behaviors (Papenfort & Bassler 2016). QS can be divided into four stages (Sifri 2008; Papenfort & Bassler 2016): (1) production of the autoinducer or chemical signals from bacterial cells; (2) release of these chemicals into the surrounding environment either actively or passively; (3) recognition of these signals by cognate receptors once they reach at threshold level; and (4) the production of specific protein as a result of gene expression. These auto-inducers or pheromones contain specific receptors on their surface which bind sensitive proteins and carry out key biological processes such as transcription and translation (Waters & Bassler 2005; Wuster & Babu 2007). Quorum quenching (QQ) is a process of preventing the action of QS and thus QS and QQ are two antagonistic processes which may co-exist and play an important role in coordinating community behaviors (Huang et al. 2016). Kim, Lee et al. (2009) studied the link between microbial QS and the propensity for biofilm formation and reported that 60% of bacteria that attached to fouled membrane surfaces contributed towards biofilm formation via intra- and inter-species communication, through the autoinducer N-acylated homoserine lactones. Interestingly, bacteria inhibiting QS decrease biofilm formation in other bacterial species (Christiaen et al. 2014). Similarly, Golberg et al. (2013) reported that coral-associated bacteria having QQ properties were inhibitory against biofilms formed by *P. aeruginosa* and *Acinetobacter baumannii*.

There is a great deal of interest among biofilm researchers in exploring the genetic changes that trigger the transition from planktonic to biofilm growth. The stepwise development of a biofilm involves a series of regulated and coordinated patterns of gene expression and protein synthesis. Two-component systems and extracytoplasmic functions are viewed as the main signaling mechanisms utilized by bacteria to sense and interpret environmental signals into adaptive mechanisms (Bordi & De Bentzmann 2011). However, some members of a phylogenetically diverse group of bacteria possess a one-component

system for signal transduction, where input and output domains are located on a single polypeptide chain (Ulrich et al. 2005; Lyon 2015). In the following sections, some molecular mechanisms involved in biofilm formation are reviewed, with particular emphasis on c-di-GMP, c-di-AMP, the TA module and a novel transcriptional regulator BolA.

Role of c-di-GMP in biofilm formation

A variety of mechanisms are involved in bacterial movement, but most, if not all, motile bacteria move with flagella, propeller-like appendages (Jarrell & McBride 2008). Bacterial motility and biofilm formation are often considered as oppositely regulated and antagonistic (for a review see Kolter & Greenberg 2006). A well-known mechanism of biofilm formation in bacterial cells is the inactivation of flagella and the activation of EPS production and fimbriae (Dressaire et al. 2015). Many mechanisms regulate the movement of flagella at either the genetic or functional level. At the level of gene expression, flagella regulating genes are divided into three hierarchies: class I, II and III and in some cases class IV also (Macnab 1992; Liu & Matsumura 1996). At the functional level, bacterial movement is controlled by chemotaxis. Bacteria use chemotaxis to detect more favorable environments (for reviews see Porter et al. 2011; Sourjik & Wingreen 2011).

There are many bacterial transcription factors involved in modulating the switch from a planktonic to a sessile lifestyle. A small cytoplasmic nucleotide signaling molecule, c-di-GMP, is known to act as a key regulator for bacterial transition from a planktonic form to the biofilm mode (Römling et al. 2005; Schirmer & Jenal 2009; Bassis & Visick 2010; Zorraqino et al. 2013). The two protein domains GGDEF and EAL are involved in the turnover of this nucleotide and thus decide its cellular concentration; GGDEE stimulates its production while EAL degrades the production of this nucleotide (Paul et al. 2004; Simm et al. 2004; Ryjenkov et al. 2006). The opposing action of the two molecules, diguanylyl cyclase (DGC), which produces c-di-GMP from the condensation and cyclization of the two GTPs, and phosphodiesterases A (PDEA), which mediates its degradation, controls the level of c-di-GMP (Ryan, Fouhy, Lucey, & Dow 2006; Hengge 2009; Schirmer & Jenal 2009). c-di-GMP is degraded into the two molecules of GMP by the action of phosphodiesterase enzymes which contain an EAL or HD-GYP amino acid motif (Simm et al. 2004; Ryan, Fouhy, Lucey, Crossman et al. 2006). Environmental cues controlling the activities of DGC and PDEA include the presence of oxygen, light or antibiotics, nutrient starvation or an oxidation-reduction state (Jenal & Malone 2006; Hengge 2009). It is also well established that a high concentration of c-di-GMP inhibits

Table 1. Names of all the proteins linked directly or indirectly with c-di-GMP and involved in biofilm formation or dispersal in various bacterial species.

Protein name	Annotation	Organism(s)	Reference(s)
FleQ	A c-di-GMP-responsive transcriptional factor which serves as a positive regulator of flagellar promoters and also regulates EPS producing genes	<i>P. aeruginosa</i> and <i>P. putida</i>	Hickman & Harwood 2008; Barraquet & Harwood 2015; Jiménez-Fernández et al. 2016
VpsT and VpsR PilZ domain protein YcgR	A master regulator for biofilm formation which inversely controls EPS production and motility C-di-GMP binding protein which inhibits motility and chemotaxis by acting as a molecular brake on flagellar motors C-di-GMP dependent transcriptional activator which regulates the formation of type 3 fimbriae and subsequently biofilm formation	<i>V. cholerae</i> <i>E. coli</i> and <i>Salmonella</i>	Krasteva et al. 2010 Ryjenkov et al. 2006; Paul et al. 2010
MrkH	AlgR regulates MuCR by binding to the <i>muCR</i> promoter region and controls its activity. Over-expression of <i>muCR</i> gives rise to c-di-GMP synthesis, which controls biofilm formation. It is a key transcriptional regulator required for the expression of type IV pilii	<i>Klebsiella pneumoniae</i>	Tan et al. 2015
AlgR	A c-di-GMP dependent biofilm dispersal mediator. It increases motility, extracellular production and decreases EPS	<i>P. aeruginosa</i>	Kong et al. 2015
BdcA (formerly YgiJ)	C-di-GMP dependent stator complex which controls biofilm formation by the repression of swarming motility	<i>E. coli</i>	Ma et al. 2011
MotAB stator complex	Pseudomonas, <i>Xanthomonas</i> , and other organisms that encode two stator systems.	<i>Pseudomonas</i>	Kuchma et al. 2015
FltA	FltA is a c-di-GMP binding transcription factor which controls biofilm formation by the deactivation of the flagellar biosynthesis regulon	<i>V. cholerae</i>	Srivastava et al. 2013
BcsA and YcgR	c-di-GMP receptors which control biofilm formation. BcsA binds with c-di-GMP and produces cellulose, major exopolysaccharide of the biofilm matrix, and YcgR inhibits motility	<i>Salmonella</i>	Zorraquino et al. 2013
FlgZ	FlgZ is a cytoplasmic protein with a predicted PilZ domain. It binds with c-di-GMP and controls flagellar gene <i>flgZ</i>	<i>Pseudomonas</i>	Martínez-Granero et al. 2014
ScrC	ScrC is a cytoplasmic membrane protein which regulates c-di-GMP. It contains both domains GGDEF and EAL and thus plays a role in biofilm formation by regulating the formation and degradation of c-di-GMP	<i>Vibrio parahaemolyticus</i>	Ferreira et al. 2008
MshE	A novel c-di-GMP protein which plays an important role in biofilm formation by inhibiting the flagella related motility and pilus production	<i>V. cholerae</i> and <i>P. aeruginosa</i>	Roelofs et al. 2015
PilZ domain protein Peld	c-di-GMP binding protein encoded by one of the genes present in the <i>pel</i> operons. It plays a role in biofilm formation by the production of extracellular PEL polysaccharide	<i>P. aeruginosa</i>	Lee et al. 2007
PilZ like protein TDE0214	A PilZ-like c-di-GMP binding protein which regulates motility, chemotaxis and biofilm formation. The TDE0214 mutants form poor biofilms	Treponema denticola (the spirochete cells) <i>P. fluorescens</i>	Bian et al. 2013
LapD	LapD is a c-di-GMP binding protein that binds c-di-GMP via a degenerate c-di-GMP phosphodiesterase (EAL) domain. It is required for adhesion to a surface and biofilm formation	<i>S. enterica</i>	Newell et al. 2008
BceE	BceE binds with c-di-GMP and results in maximal cellulose production, which is an important component of biofilms in <i>Enterobacter</i> species	<i>B. cenocepacia</i>	Fang et al. 2014
Bcam1349 protein	A transcriptional regulator that binds c-di-GMP and regulates biofilm formation via regulating the production of cellulose and fimbriae in <i>Burkholderia cenocepacia</i> in response to the level of c-di-GMP	<i>B. cenocepacia</i>	Fazli et al. 2011

cell motility and activates EPS production and thus promotes sessile life, while lower concentrations result in a planktonic mode of life (Hickman & Harwood 2008). The importance of c-di-GMP in bacterial physiology can be understood from the fact that there may be numerous DGC and PDEA in even a single bacterial strain, for instance, in *Escherichia coli* there are a total of 12 proteins having a GGDEF domain; of these, 10 have an EAL domain, and seven have both EAL and GGDEF domains in one single polypeptide (Hengge et al. 2015).

c-di-GMP is an ubiquitous signaling molecule that is known to be present in > 80% of bacteria. Despite its role in many cellular mechanisms, particularly in switching from motility to a biofilm state, its complete signaling mechanism in a number of bacteria is still obscure. Its role has been recently explored in various bacteria, including *Legionella pneumophila* (Pécastaings et al. 2016), *Azospirillum brasilense* (Ramírez-Mata et al. 2016) and *Klebsiella pneumoniae* (Tan et al. 2015; Guilhen et al. 2016).

There are, however, several known transcription factors that are linked to c-di-GMP and control genes involved in either EPS production, cell motility or both. The list of known c-di-GMP responsive transcription factors or those that regulate c-di-GMP and biofilm formation is given in Table 1. All transcription factors work on the same principle of inhibiting motility by binding to the specific sites of c-di-GMP. c-di-GMP can reduce motility by downregulating the genes involved in flagellar expression. For instance, in *P. aeruginosa*, c-di-GMP reduces motility by downregulating either flagellar expression or by interacting with flagellar motor function. In *P. aeruginosa*, biosynthesis of the polar flagellar genes is controlled by a cascade, which is directly headed by a transcriptional regulator (FleQ). This protein regulates many genes involved in flagellar production, such as *fliA*, *fliE*, *fliL*, and *fleSR* (Jyot et al. 2002). The activity of FleQ is regulated by two known mechanisms: (i) by the interaction of an anti-activator (FleN, putative ATP/GTP binding protein) which downregulates the flagellar genes by binding to FleQ and inhibiting its ATPase activity (Dasgupta & Ramphal 2001); and (ii) by c-di-GMP binding to FleQ and dampening its ability to initiate downstream flagellar gene expression. Interestingly, this effect is more pronounced in the presence of FleN (Baraquet & Harwood 2013). Matsuyama et al. (2016) elegantly provided some mechanistic insights into c-di-GMP-dependent control of FleQ (biofilm regulator) from *P. aeruginosa*.

Apart from its role in downregulating genes involved in flagellar regulation (as mentioned above), the role of c-di-GMP in interfering with the flagellar rotation by affecting the flagellar machinery in *P. aeruginosa* is also pronounced. The flagellar machinery consists of rotor,

stator, flagellar hook and filament proteins. *P. aeruginosa* encodes two stator complexes, MotAB and MotCD, which regulate the function of its single polar flagellum. MotCD drives swarming motility, while the MotAB stator does not support swarming. It has been hypothesized that when the level of c-di-GMP is high, the MotAB stator may repress motility by replacing or displacing the stator MotCD (Kuchma et al. 2015). However, the exact mechanism of c-di-GMP's interaction with the two stators is not well understood. Apart from controlling motility, c-di-GMP also regulates the biofilm matrix of *P. aeruginosa*. When intracellular levels of c-di-GMP are high, FleQ binds with c-di-GMP and depresses the expression of *pel* exopolysaccharide genes (from the *pel* operon) (Baraquet et al. 2012) and the *cdrAB* gene, encoding a cell surface adhesion (Baraquet & Harwood 2016), both of which contribute towards forming the biofilm matrix.

Recently Jiménez-Fernández et al. (2016) provided evidence that FleQ also serves as the master regulator of the flagellar cascade in *Pseudomonas putida*, as a *fleQ* mutant was highly defective in flagella-based swimming and swarming motility. It was further proposed that a regulatory cascade, described in *P. putida*, is reminiscent of the regulatory cascade described in *P. aeruginosa* which controls flagellar motility (described above) and its activity is antagonized by c-di-GMP. Thus, it seems that the level of c-di-GMP controls biofilm formation via FleQ which acts as a transcriptional regulator of flagellar genes and EPS (Pel and adhesions).

Similarly, a PilZ domain protein, YcgR, also known as a flagellar brake, is a c-di-GMP binding protein in *Escherichia coli* and *Salmonella* which modulates the flagellar rotation by interacting with flagellar switch complex proteins, FliG and FliM. The binding of c-di-GMP to YcgR is important for the YcgR-regulated motility inhibition. YcgR binds to both FliG and FliM in the absence of any external effector and these bindings increase with the concentration of c-di-GMP (Paul et al. 2010).

Role of *BolA*, a novel transcriptional regulator in biofilm formation

The gene *bolA* is widely distributed in nature and is present in most Gram-negative bacteria. It was initially characterized in *E. coli* as a stationary-phase morphogene that results in round colony morphologies upon its over-expression regulated by the σ^S -dependent *gearbox* promoter *bolA1p* (Aldea et al. 1988). However, later it was revealed that its role is not only confined to stationary phase as its expression (σ^S -independent) is triggered by exposure to various stress conditions like acidic stress, heat shock, oxidative stress, osmotic stress and sudden carbon starvation during early exponential growth phase (Santos

et al. 1999). These observations led to the idea that the morphogene *bolA* may have a role in biofilm formation as many of these conditions prevail in biofilms. However, the first compelling evidence of the role of the *bolA* gene in biofilm formation was revealed when Vieira et al. (2004) reported that the deletion of this gene decreases biofilm formation in *E. coli* and its over-expression gives rise to biofilm formation. The role of the gene *bolA* in biofilm formation has been reported in *E. coli* as well as *Pseudomonas fluorescens* (Koch & Nybroe 2006). Now, BolA is considered to be a new transcription factor that regulates biofilm formation by directly binding to the promoter regions of many important genes involved in biofilm formation (Dressaire et al. 2015). Evidence has been presented that it directly regulates many cellular functions including cell motility, and flagellar and curli synthesis pathways (Dressaire et al. 2015). ChIP-seq and transcriptomic profiles have indicated that BolA interacts with DNA sequences and represses several genes involved in the production of flagella (*fliC*, *fliF*, *fliG*, *fliN*, *fliM*) and flagellar apparatus-like hook (*fliD*, *fliJ*) by downregulating the flagellar master regulator FlhDC. BolA plays a key role in initial bacterial attachment by promoting the production of curli and fimbria-like adhesions and expression of genes positively involved in the initial attachment.

BolA also increases expression of genes involved in producing different components of the biofilm matrix, e-DNA, proteins, sugar, lipopolysaccharide and cellulose. On the other hand, it also induces the expression of genes involved in the tricarboxylic acid cycle (TCA), such as *sucA*, *ybjJ*, *icd*, *sucB*, *sucC*, *sdhB*, *sdhC* and *gltA*. Enhanced TCA activity is advantageous for nutrient- and oxygen-starved cells in biofilms as it will enhance acetate and amino acid catabolism and the efficient consumption of traces of oxygen (Gaupp et al. 2010). It is likely that this protein may have a role in the biofilm regulatory network in many other Gram-negative species.

Biofilm formation by the TA module

TA systems are small genetic elements, comprised of two genes (organized in a single operon), a toxin gene which disrupts essential cellular processes and its cognate anti-toxin (either RNA or a protein) that prevents toxicity (Van Melderen & De Bast 2009). Type I and type III anti-toxins are RNA in nature while type II anti-toxin is protein in nature. Type I anti-toxins or the antitoxin antisense RNAs prevent the translation of toxin RNAs. Similarly, type III anti-toxins are also RNA in nature and stop or reduce the activity of toxins. Type II anti-toxins inhibit the activity of toxins (Leplae et al. 2011). Most of the toxin and anti-toxins form a complex with each other, which neutralizes the toxicity of the toxin. Also, both anti-toxin and the complex

can bind to the regulatory region of the operon and inhibit its transcriptional function. However, in the type IV TA system, the anti-toxin CbeA interferes by binding the targets of the toxin CbtA rather than forming a complex. Similarly, in the type V system, the antitoxin (GhoS) acts as an endoribonuclease and specifically cleaves the toxin (GhoT) mRNA (Wen et al. 2014).

The toxins in the TA module are proteins and impede cellular functions by interfering with DNA replication, cell wall synthesis, membrane disruption, adenosine triphosphate (ATP) synthesis, cytoskeletal polymerization and phage infections (see a review by Unterholzner et al. 2013). It has now become evident that these systems are important genetic modules that are ubiquitously distributed in the bacterial genome and play a significant role in various functions including biofilm formation. In *E. coli* there are around 38 TA systems (Tan et al. 2011), while in *Mycobacterium tuberculosis* there are 79 (Sala et al. 2014). MqsR/MqsA was reported as the first pair of the TA system in *E. coli* with a link to biofilm formation. The gene *mqsR* (responsible for motility quorum-sensing) was induced in a transcriptomic profile of *E. coli* during biofilm formation as compared with the exponential growth phase (Ren et al. 2004). Later studies clearly demonstrated the role of MqsR/MqsA in motility, biofilm formation and persistence in *E. coli* (Barrios et al. 2006). Recently, the role of MqsRA in positively regulating biofilm formation in *Xylella fastidiosa* has also been reported (Merfa et al. 2016).

In further studies, it was found that external stress affects expression of genes controlled by MqsA. For instance, MqsA represses the master regulator of stress (*RpoS*), resulting in a decreased production of c-di-GMP which ultimately leads to increased motility and a reduced biofilm. However, under oxidative stress MqsA is degraded by the Lon protease, which results in the activation of *RpoS*, leading to suppression of motility or biofilm formation, as shown in Figure 4 (Wang et al. 2011). These findings are in accordance with a study of Soo and Wood (2013) from which it was concluded that in the absence of any stress, MqsA increases motility by increasing the transcription of *flhD*, which encodes FlhDC, a master regulator for motility, and repressing the master regulator CsgD, which regulates the curli genes *csgA* and *csgB* and cellulose production, partly through *rpoS* inhibition. Under stress conditions, MqsA is degraded by Lon protease (Wang et al. 2011), which results in the increased production of the toxin MqsR. The degradation of MqsA leads to de-repression of the stress response regulator *rpoS*, which results in the activation of CsgD and inhibition of *flhD* via c-di-GMP. These consequences ultimately lead to biofilm formation. The activation of CsgD via *RpoS* may itself regulate motility. For instance, CsgD activates *ihfB*, which

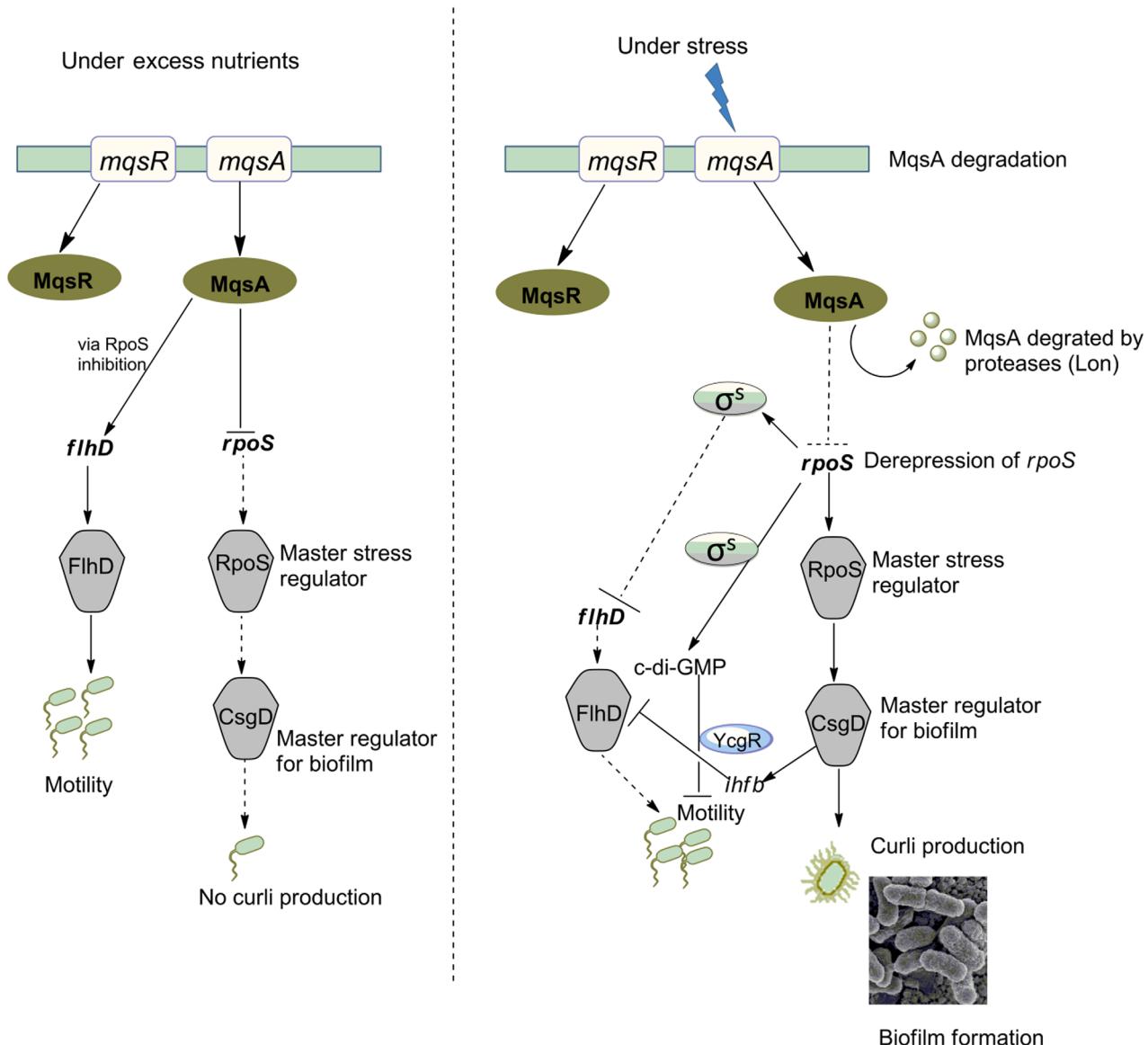


Figure 4. The role of the toxin MqsA of the MqsRA toxin–antitoxin system in motility and biofilm formation in *P. aeruginosa*. When there is no stress the toxin MqsA inhibits the master stress regulator RpoS. When RpoS is repressed, there will be no c-di-GMP production to inhibit motility and also the master regulator for biofilm, CsgD, will not be activated; thus there will be no production of curli, leading to motility. Under stress conditions, the toxin MqsA will be degraded by protease and will no longer be able to repress RpoS. RpoS will increase the sigma S factor which will give rise to c-di-GMP, leading to inhibition of motility via YcgR. Sigma S factor will also repress *flhD* which encodes FlhD, master regulator for motility (Wang et al. 2011; Soo & Wood 2013). Additionally CsgD will also activate *ihfb* which will further inhibit FlhD (Yona-Nadler et al. 2003).

reportedly represses FlhD (Yona-Nadler et al. 2003). In the light of the above discussion, Figure 4 shows how MqsA controls biofilm formation and motility under stress or excess nutrients, respectively.

Further evidence of the role of TA systems in biofilm formation was accumulated from studies on *E. coli* Δ5 which had five TA systems (MazF/MazE, RelE/RelB, YoeB/YefM, YafQ/DinJ, and ChpB) deleted. It was observed that the mutant strain lacking these five systems showed reduced biofilm formation after 8 h with the differential expression of only one gene *yjgK*. Further transcriptome profiling confirmed that the gene *yjgK* encodes the protein

YjgK, which represses the fimbria gene (*fimA*) at 8 h and it is repressed by these TA systems (Kim, Wang et al. 2009).

It could be inferred from the results of previous investigations that controlled bacterial cell death and lysis are associated with biofilm formation and development (Rice et al. 2007; see a review by Allocati et al. 2015). Now, there is an increasing recognition that biofilm formation via the TA module is facilitated by cell death and the release of e-DNA which serves as a main component of biofilms. Interestingly, the five TA systems mentioned above have been reported to be involved differently in cell death in *E. coli* strain MC4100 (Kolodkin-Gal et al. 2009). Two of

these five TA systems, MazF/MazE (Hazan et al. 2004) and YafQ/DinJ (Liang et al. 2014), have been reported to have a role in cell death in *E. coli*. MazEF and DinJ-YafQ-mediated cell death was thought to have a possible link with biofilm formation through an unknown mechanism (Kolodkin-Gal et al. 2009). Zhao et al. (2013) demonstrated the role of the *hipBA* TA system of *E. coli* strain BW25113 in biofilm formation through the release of e-DNA. In *E. coli*, *hipBA* is a type II TA system that contains the gene *hipA*, which codes for the HipA toxin, and the gene *hipB* which encodes the antitoxin HipB. The deletion of the *HipBA* TA system in *E. coli* BW25113 resulted in reduced biofilm formation. It was concluded that the activation of *hipA* significantly enhances biofilm formation through the production of e-DNA, as a result of cell lysis (Zhao et al. 2013). Wood and Wood (2016) have recently described a unique role for a novel type II TA system HigB/HigA in biofilm formation in *P. aeruginosa*. They demonstrated that the toxin HigB reduces virulence factors (pyocyanin production) and biofilm formation. Reduced biofilm formation occurs as a result of a decrease in the production of pyocyanin, which can be linked to less cell lysis and therefore less e-DNA release. Pyocyanin promotes e-DNA release through cell lysis in *P. aeruginosa* (Das & Manefield 2012). Toxins produced by certain TA systems may not have killing effect on bacterial cells. HicAB has recently been characterized as a new type II TA system in *P. aeruginosa* in which the toxin HicA has bacteriostatic rather than bactericidal effect on growth, and interestingly this toxin had no effect on biofilm formation, which further supports the role of lysis-based e-DNA in biofilm formation (Li et al. 2016). For details on the role of the TA module in persistence, biofilm formations, and pathogenicity, see a review by Wen et al. (2014).

Role of cyclic di-AMP in biofilm formation

c-di-AMP is a novel and emerging second nucleotide signaling messenger that controls a myriad of physiological aspects of bacterial life including biofilm formation. The production of c-di-AMP is regulated by the opposing action of two enzymes: diadenylate cyclase (DAC) that synthesizes this molecule from two molecules of ATP or ADP and phosphodiesterase (PDE), that degrades this molecule into pApA or AMP (Huynh et al. 2015). The complete mechanism of its formation and the currently known receptor proteins and pathways controlled by c-di-AMP have been reviewed by Corrigan and Gründling (2013).

The first report of c-di-AMP production from bacterial cells was in 2008 during the study of the *B. subtilis* checkpoint protein (DNA integrity scanning protein A, DisA) (Witte et al. 2008). It was also reported as being secreted in the

host cells cytosol by the intracellular pathogen *Listeria monocytogenes* (Woodward et al. 2010). The role of c-di-AMP in biofilm formation has been reported in various bacteria. Recently, Peng and his colleagues (Peng et al. 2016) unraveled the mechanistic role of c-di-AMP in biofilm formation in *Streptococcus mutans*. This bacterium along with other oral bacteria facilitates the formation of dental plaque through the production of water soluble and insoluble glucans. The enzyme GtfB plays a critical role in biofilm formation (Yamashita et al. 1993). It was demonstrated that c-di-AMP controls *S. mutans* biofilm formation by mediating *gftB* expression through the binding of the c-di-AMP receptors CabPA (containing TrkA_C domains) to VicR, a putative response regulator that mediates the expression of *gftB* (Peng et al. 2016). In contrast to the results of Peng et al. (2016), suggesting an increase in biofilm formation as a result of elevated level of c-di-AMP, Cheng et al. (2016) reported an increase in the production of extracellular polysaccharides in the absence of c-di-AMP in *S. mutans*.

Ono et al. (2014) showed that *P. aeruginosa* mutant strains ($\Delta cpdA$) that lacked the ability to degrade c-di-AMP were more hydrophilic than the wild type. These results demonstrate that c-di-AMP may have a role in biofilm formation by influencing the cell attachment by altering cell hydrophobicity.

c-di-AMP accumulation also inhibits biofilm formation in *B. subtilis* by affecting the expression of genes controlled by the master regulator SinR. It was suggested that c-di-AMP does not affect the cellular concentration of SinR, but probably affects the activity of SinR by interfering with the interaction between SinR and its antagonists (Gundlach et al. 2016). More knowledge is needed on the mechanistic role of c-di-AMP in regulating biofilms, eg by influencing different transcriptional regulators involved in motility or matrix production.

The motile-to-sessile switch in *P. aeruginosa* is affected by various transcriptional regulators

P. aeruginosa is a Gram-negative rod shaped pathogenic bacterium. It causes acute or chronic opportunistic infections in immunocompromised humans or critically ill patients (Wilson & Dowling 1998). The infective nature of this bacterium is associated with its motility and its propensity for biofilm formation (Murray et al. 2010). *P. aeruginosa* is motile via flagella and type IV pili (T4P); flagella help in near-surface swimming and surface-anchored spinning while, pili-mediated movement involves horizontally oriented crawling and vertically oriented walking (Conrad et al. 2011). The first step in biofilm formation is the reversible attachment of a bacterium to a surface followed by the secretion of EPS. During

the initial stage of biofilm formation (a switch from planktonic form to sessile life), both flagella and pili play a key role (Barken et al. 2008), as deleting or altering any of these appendages leads to impairment or deficiencies in cell surface attachment and growth. The role of c-di-GMP in regulating biofilm formation in *P. aeruginosa*, mainly by exerting its effect on FleQ's regulatory roles, has been previously discussed in several reviews (Ha & O'Toole 2015; Valentini & Filloux 2016). Therefore the following discussion has focused on biofilm formation in *P. aeruginosa* under the influence of all known transcriptional regulators apart from the role of c-di-GMP.

Many Gram-negative bacteria have the GacS/GacA two-component system (TCS) (or its homologs) which positively regulates the expression of target genes involved in regulating motility or the production of extracellular products and secondary metabolites *via* small regulatory RNAs. Small RNAs (sRNAs) are non-coding RNA molecules (containing 18 to 30 nucleotides) that can regulate gene expression through post-transcriptional and chromatin-dependent gene silencing (Carthew & Sontheimer 2009).

This TCS consisting of sensory kinases and response regulators has evolved to be a successful regulatory tool for the adaptation of bacteria to rapidly changing environments. In *P. aeruginosa*, transcription of two genes, *rsmY* and *rsmZ*, encoding sRNAs is regulated by a TCS (GacS/GacA). The opposing action of the two sensory kinases, RetS and LadS, is involved in regulating the GacS/GacA signal-transduction pathway through modulation of the phosphorylation state of GacS/GacA (Bencic et al. 2009). RetS negatively controls the expression of the genes *rsmY* and *rsmZ* while LadS positively controls the expressions of both genes. GacA in its phosphorylated state activates the expression of the sRNAs which antagonize the activity of RsmA (Ventre et al. 2006; Goodman et al. 2009). It is known that GacA-dependent sRNA encoded genes (*rsmY/rsmZ*), which are involved in the regulation of the quorum sensing signal N-butanoyl-homoserine lactone (C4-HSL) in *P. aeruginosa*, are also involved in the regulation of motility and biofilm formation (Heurlier et al. 2004; Kay et al. 2006). RsmA regulates swarming motility by positively modulating the synthesis of flagella, T4P and Type III secretion systems (T3SS) (Bencic & Lory 2009). On the other hand, RsmA downregulates the expression of genes required for the production of Pel and Psl polysaccharides, which serve as a structural scaffold and play a key role in maintaining biofilm integrity (Irie et al. 2010). Bencic et al. (2009) reported that in *P. aeruginosa* the GacS/GacA system directly regulates only two genes *rsmY* and *rsmZ*. So, the expression of hundreds of genes regulated by the RetS/GacS/GacA system is controlled by sRNA (RsmY and RsmZ) dependent modulation of

RsmA. Figure 5 shows a general mechanism of biofilm formation in *P. aeruginosa* by the TCS GacS/GacA and two sensor kinases RetS and LadS. The figure shows that GacA in its phosphorylated state activates two sRNAs (RsmY and RsmZ), which repress the activity of the translational repressor RsmA. Titration of RsmA decides the fate of bacterial cells; high levels lead to motility while low levels lead to biofilm formation.

RetS forms heterodimers with GacS and blocks its auto-phosphorylation, which ultimately blocks the phosphorylation of the response regulator GacA leading to reduced sRNA expression (Bencic et al. 2009; Chambonniere et al. 2016). RsmA lacking RsmZ promotes acute virulence by producing T4P and T3SS, while discouraging chronic infections or repressing the expression of Psl-fiber matrix or Pel (alginate). RetS and GacS may form homodimers as a result of an external signal which results in the auto-phosphorylation of GacA and phosphorylation of GacA, which ultimately leads to the expression of genes in biofilm formation and chronic infection (Goodman et al. 2009).

Apart from RetS and LadS, there are several other response regulators, which control the levels of sRNAs. For instance, Bordi et al. (2010) reported a histidine phosphotransfer protein HptB located upstream of GacA that also modulates sRNA levels. HptB also interacts with GacA component in the same way that RetS does, but unlike RetS, which controls the expression of both genes *rsmY* and *rsmZ*, it exclusively regulates *rsmY* expression. Also it was suggested that it is likely to act through a phosphorelay rather than forming a heterodimer with GacS as RetS does. These differences clearly show that RetS and HptB are not cognate partners. In research conducted by Petrova and Sauer (2010), it was demonstrated that the modulation of different levels of *rsmZ* or the decay of *rsmZ* RNA levels at the post-transcriptional level were also dependent on BfiSR, a novel two-component system which controls the suppression of *rsmZ* through RNase G (CafA) under biofilm growth conditions (Figure 6). Additionally, MvaT and MvaU are histone-like nucleoid structuring proteins (H-NS), which preferentially associate with the AT-rich chromosomal region (Castang et al. 2008). It has been reported that a region of DNA near *rsmZ*, not *rsmY*, associates with these two proteins and interestingly MvaT repressed the transcription of *rsmZ* by binding to its promoter; however, it did not affect the expression of *rsmY* (Bencic et al. 2009). Wang et al. (2014) reported a novel transcriptional factor BswR, a xenobiotic response element-type transcriptional regulator, which plays a key role in the regulation of motility and biofilm formation in *P. aeruginosa* by counteracting the repressor activity of MvaT. It reportedly also regulates the biogenesis of flagella by binding to the promoter of *rsmZ* as over-expression of

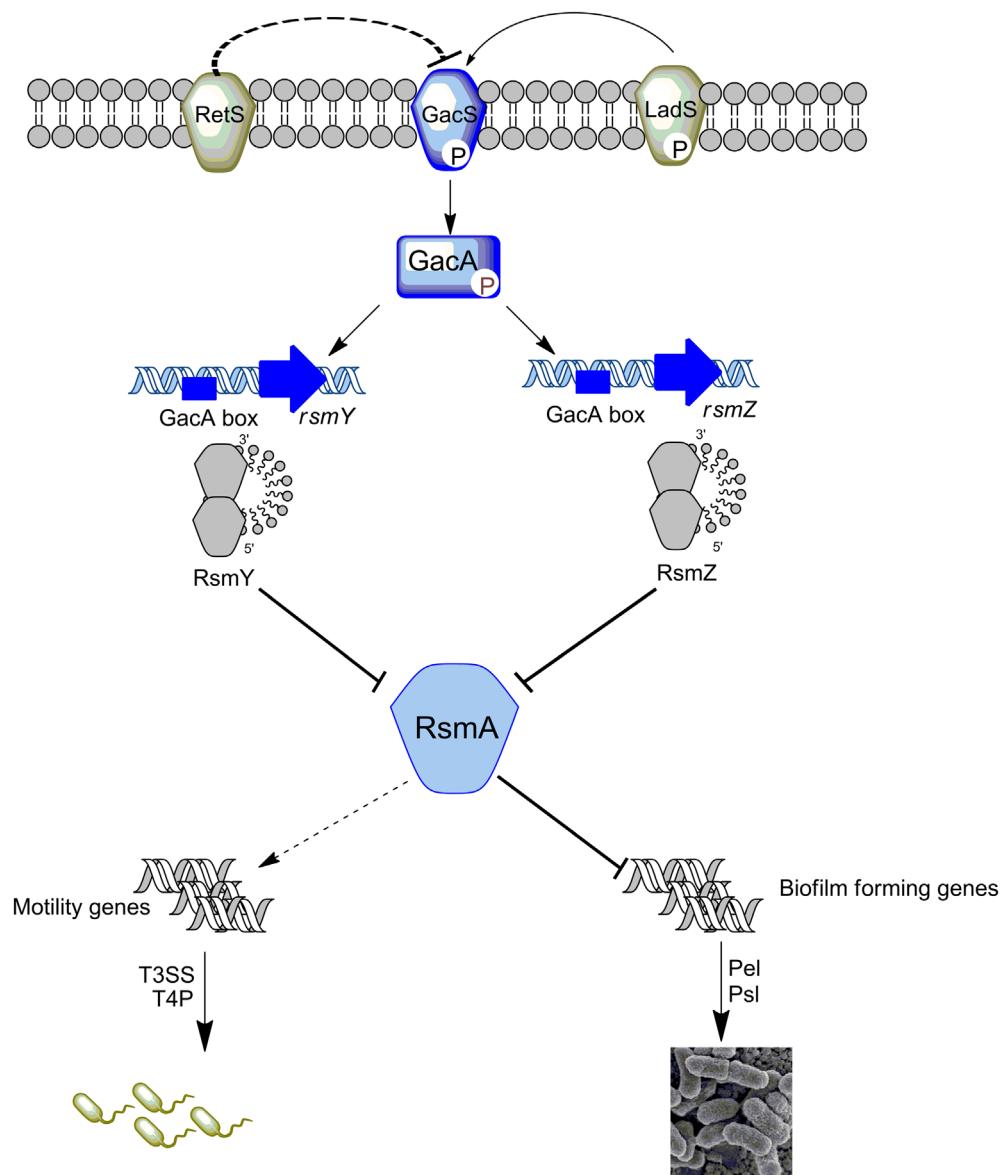


Figure 5. The role of the two component system GacS-GacA in the motile to sessile switch in *P. aeruginosa*. GacA in its phosphorylated state activates two small RNAs (RsmY and RsmZ), which repress the activity of translational repressor RsmA. Titration of RsmA decides the fate of bacterial cells; high quantity leads to motility (Bencic & Lory 2009).

this protein enhances the expression of *rsmZ* and deactivates RsmA, which hinders the expression of Type IV pili (T4P) and flagellar genes (Wang et al. 2014). Zhu, Zhao et al. (2016) reported a gene modulator, TspR which was shown to modulate the quantity of T3SS through either two sRNAs or a master regulator ExsA. They also reported that the activity of TspR was controlled by RetS (Figure 6).

Surprisingly, the levels of sRNAs can control biofilm formation; high levels of sRNA can increase bacterial attachment; however, biofilm formation (post-attachment) increases with reduced levels of sRNAs, particularly RsmZ, compared to its expression in planktonic cells (Petrova & Sauer 2010). This suggests that a fine-tuning of sRNA levels decides the fate of bacterial cells.

Thus, biofilm formation in *P. aeruginosa* can be divided into two phases: initial attachment and post attachment. It can be hypothesized that, initially, bacterial colonization on a surface is characterized by expression and modulation of genes and proteins (including LadS, GacSA, HptB) that promote initial attachment by increasing the levels of sRNA (Petrova & Sauer 2011). Colonizing bacteria, perhaps, also express genes responsible for the production of BswR, which is particularly involved in repressing the transcriptional activity of MvaT and enhancing the expression of *rsmZ* (Wang et al. 2014). Thus, after attachment bacteria need different proteins (BfiS and BfiR through CafA (Petrova & Sauer 2011) and possibly MvaT and MvaU) to reduce the production of sRNAs and

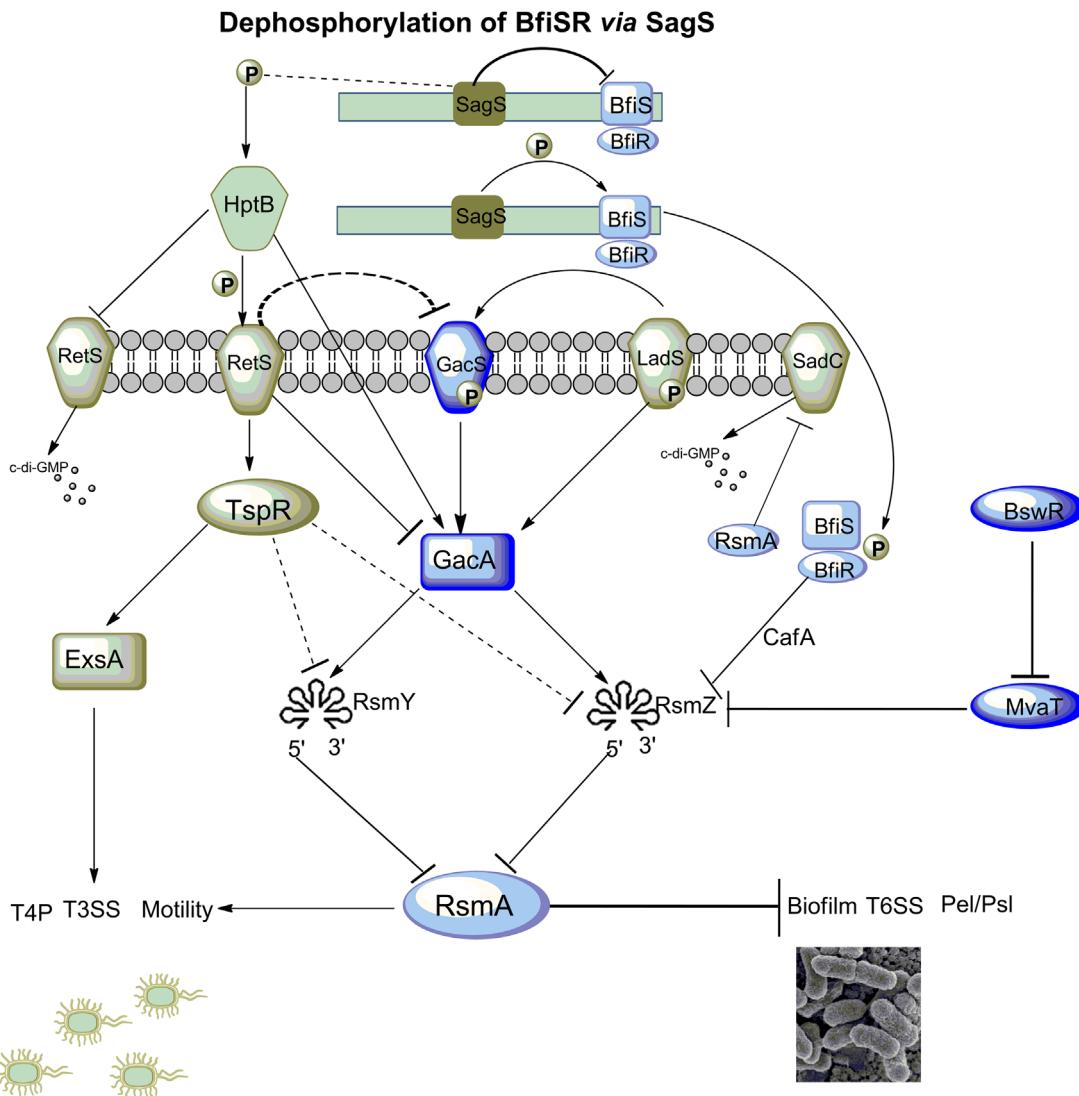


Figure 6. Model for the role of various transcriptional regulators involved in prior to and following the surface attachment in *P. aeruginosa*. Before biofilm formation LadS, GacSA (Petrova & Sauer 2010) and HtpB (Bordi et al. 2010) help bacterial cells to produce high levels of small RNAs required for initial attachment (Petrova & Sauer 2010). The activity of MvaT is presumably repressed by BswR during the initial attachment (Wang et al. 2014). For surface growth and subsequent biofilm formation levels of sRNAs (particularly RsmZ) are reduced by the activation of BfiSR through SagS dephosphorylation, phosphoryl group transfer to BfiS from SagS (Petrova & Sauer 2011) and BfiSR represses RsmZ via CafA at the post-transcriptional level during the biofilm development (Petrova & Sauer 2010). Reduced expression of sRNAs, particularly RsmZ, result in biofilm formation. TspR regulates bacterial motility through the activation of T3SS via ExsA and directly represses both small RNAs. The activity of TspR is regulated by RetS (Zhu et al., 2016). Cyclic-di-GMP is also involved in this Gsc/Rsm cascade and it is produced by two DGCs, SadC and HsbD. RsmA inhibits SadC (Moscoso et al. 2014), while HptB inhibits HsbD (Valentini et al. 2016).

to increase surface related growth or to develop normal biofilms. Petrova and Sauer (2011) reported that SagS, one of the three cognate sensors of HptB, controls both phases (initial attachment and post attachment) of biofilm formation in *P. aeruginosa*. It was demonstrated that SagS interacts directly with BfiS and indirectly with BfiR and modulates sRNA levels by the phosphorylation and dephosphorylation of BfiSR during the post attachment and attachment phases, respectively (Petrova & Sauer 2011). Like SagS, the role of TspR in regulating biofilm formation and T3SS is very interesting. It negatively regulates

the transcriptional activity of both sRNAs (which results in increased post-attachment biofilm formation) on one hand, and on the other hand, it activates a master regulator ExsA, which enhances the production of T4P and T3SS (Zhu, Zhao et al. 2016). In the light of the above discussion, Figure 6 shows how all proteins and transcriptional regulators affect the levels of sRNAs and decide the fate of bacterial cells to switch to motility or the biofilm mode of life.

The above discussion has focused on transcriptional regulators other than c-di-GMP. However, it is important

to mention that this cascade (GacSA-RsmYZ-RsmA) also involves c-di-GMP through SadC, a DGC. The production of SadC is repressed by RsmA (Moscoso et al. 2014). However, over-expression of SadC in *P. aeruginosa* PAO1 was reported to be linked with inhibited motility and enhanced synthesis of Psl (Zhu, Liu et al. 2016). Recently, a novel DGC (HsbD) has been reported to be located upstream of RsmA and ultimately SadC. It is directly controlled by the HptB pathway and provides insights into a novel pathway elucidating how c-di-GMP is orchestrated in this bacterium through this cascade (Valentini et al. 2016). These two c-di-GMP controlling DGCs are shown in Figure 6.

Biofilm formation in *B. subtilis*, a paradigm of Gram-positive bacterium

Over the last decade *B. subtilis*, a Gram-positive non-pathogenic microbe, has emerged as an alternative bacterium to *P. aeruginosa* to study the genetic basis of biofilm formation (Vlamakis et al. 2013). In *B. subtilis*, planktonic growth and biofilm formation are two exclusive lifestyles; each depends on different proteins and gene expression. In order to consider any aspect of biofilm formation, it is important to consider all factors and regulators that control motility and synthesis of extracellular matrix. Motility and EPS production are often oppositely regulated processes. In *B. subtilis*, a fraction of the isogenic population produces at least two of the three mandatory matrix components to form biofilm. Two extracellular components are considered as primary constituents of its biofilm matrix: (1) EPS, synthesized by the products of a 15 gene operon (*epsA-epsO* or *epsA-O*) and (2) amyloid fibers, synthesized by the protein TasA, as a result of the expression of the *tapA-sipW-tasA* operon (Branda et al. 2006). In addition to these two components, the extracellular protein BslA, encoded by the monocistronic *bslA* gene, is considered as the third component that is necessary for the biofilm matrix and is synthesized by the whole population (Kovacs & Kuipers 2011). The structurally defined bacterial hydrophobin plays an important role in the assembly of the biofilm matrix and coats the *B. subtilis* biofilm (Hobley et al. 2013). In *B. subtilis*, the gene (*hag*) controls the flagellar filament expression and it is under the control of RNA polymerase containing the alternative σ factor (σ^D). Interestingly, the cells that are ON for σ^D also show autolysin expression (LytC, LytD and LytF) and therefore concomitant expression of motility and autolysin genes is a feature of this bacterium (Chen et al. 2009) as shown in Figure 7b.

The activation of Spo0A is the most important event that takes place in the transition from planktonic growth to the biofilm mode of life or before sporulation (Figure 7a).

Spo0A, a transcriptional regulator, is a member of the response regulator family, which is activated by phosphorylation (*via* phosphorelay consisting of five histidine autokinases) and the two phosphorelay proteins Spo0F and Spo0B (Jiang et al. 2000). The activation of Spo0A relies on phosphorylation *via* the phosphorelay and is also controlled by several other complex networks, the functioning of most of which are unknown (Jiang et al. 2000). Recently, Dubnau et al. (2016) presented evidence for the role of a protein complex (YlbF, YmcA and YaaT) in supporting the phosphorylation of Spo0A in *B. subtilis*. Mutants of these genes were found to be blocked in biofilm formation, although the mechanism was not explained. This protein complex is shown in Figure 7a. However, a further report questioned the findings of Dubnau et al. (2016), as it demonstrated that the complex has no role in the phosphorelay or on the expression of Spo0A~P-directed genes ie *sinI*. This protein complex is co-conserved in those Gram-positive bacteria which even lack the phosphorelay (De Loughery et al. 2016). The possible role of this protein complex is discussed in a later part of this section.

Yan et al. (2016) reported a novel gene *comER* which plays an important role in biofilm formation and suggested that this gene may be a part of the regulatory circuit that controls Spo0A activation (Figure 7a). It was further reported that this gene controls a small check point protein (Sda) by acting upstream of the gene *sda*. The protein Sda reportedly inhibits Spo0A activation by blocking the phospho-transfer from KinA to the phosphor carrier Spo0F (Whitten et al. 2007), as shown in Figure 7a. Recently, another operon (*mstX-yugO*), negatively regulated by SinR, has been reported to be involved in biofilm formation by mediating the activation of Spo0A. It was reported that the expression of *mstX* and the downstream putative K⁺-efflux channel, *yugO*, are necessary for biofilm formation. *mstX* mediates the biofilm-inducing putative K⁺ channel *yugO* which leaks K⁺ (Figure 7a). The leakage of K⁺ results in the activation of KinC, which in turn activates Spo0A. This report was the first description of *MstX* mediated biofilm formation in *B. subtilis* (Lundberg et al. 2013).

Spo0A, in its phosphorylated state (Spo0A~P), turns on the transcription of *sinI* and represses the transition state regulator AbrB through the activation of the antirepressor AbbA, which inhibits the DNA binding activity of AbrB (Banse et al. 2008). The transition state regulator AbrB, as well as repressing the *tapA-sipW-tasA* and *eps* operons, also represses the activity of the matrix protein BslA, regulatory proteins, Abh and SlrR (see a review by Vlamakis et al. 2013) and a putative secreted protein YoaW (Hamon et al. 2006) which plays an important role in building the biofilm structure in *B. subtilis*. The

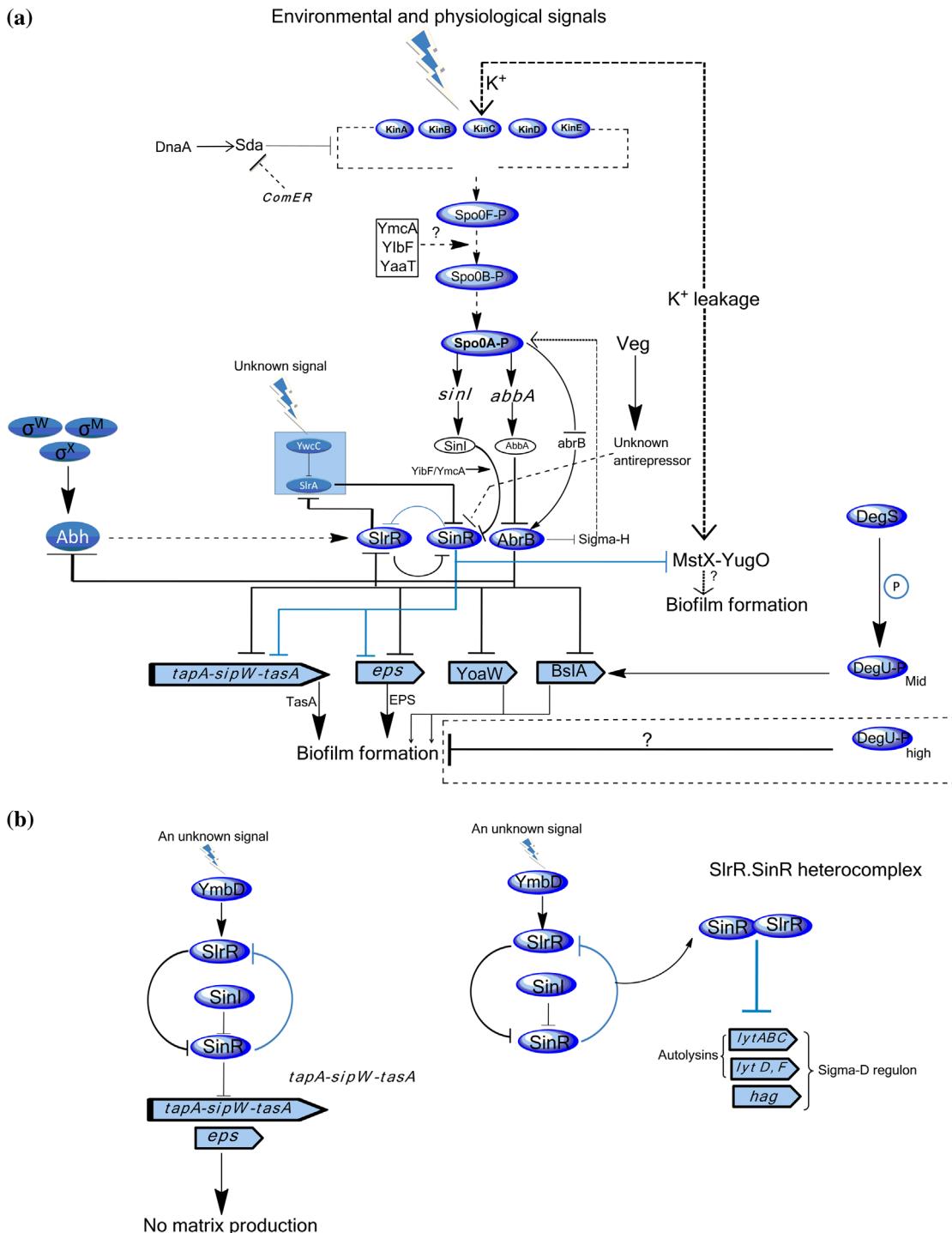


Figure 7. A detailed description of molecular mechanisms involved in biofilm formation in *B. subtilis*. The response regulator Spo0A in its phosphorylated state plays a key role in biofilm formation by controlling the transcriptional activity of *sinl* and *abbA*, which are involved in repressing SinR and AbrR (transition state regulators). Several factors have been shown to be involved in controlling the Spo0A phosphorylation, eg a novel gene *comER* (Yan et al. 2016), an operon *mstX-yugO* (Lundberg et al. 2013) and a protein complex (YmcA, YibF, YaaT) (Dubnau et al. 2016). The operons *epsA-epsO* or *epsA-O* and *tapA-sipW-tasA* form biofilm matrix, while the proteins BslA and YoaW also play a key role in biofilm matrix. It has been shown that SinR inhibits biofilm matrix production through repressing the genes involved in biofilm formation. It also inhibits motility when it forms a heterocomplex with SlrR as shown in (b) (adapted from Chai et al. 2010). YmbD, a novel factor, plays a key role in SlrR signaling through an unknown signal as shown in (b) (Diethmaier et al. 2011). The activity of SinR is repressed by Sinl, SlrA and an unknown repressor Veg. DegU and Abh are other networks which control the activity of SlrR and the biofilm surface layer protein BslA, respectively. The mechanism in this figure shows the previously described mechanism of biofilm formation in *B. subtilis* (modified with permission from Vlamakis et al. 2013), and provides the latest insights.

transcription of *abh* gene is controlled by extracytoplasmic function (ECF) σ -factors, which are involved in antibiotic formation and include σ^M , σ^W and σ^X (Eiamphungporn & Helmann 2008; Luo & Helmann 2009) (Figure 7a).

SinI is an anti-repressor; once it is activated by *Spo0A*, it binds to *SinR* (a tetrameric repressor protein) *via* protein–protein interactions and antagonizes its activity, thereby derepressing the matrix biosynthesis genes and the gene for *SlrR* (*slrR*) (Lewis et al. 1996). The two proteins *YmcA* and *YlbF* are thought to be involved in counteracting *SinR*-mediated repression by interacting with *SinI* or *SinR* (or both) (Kearns et al. 2005; Lemon et al. 2008). De Loughery et al. (2016) noticed a block in biofilm formation in the absence of the genes *ylbF*, *ymcA* and *yaaT*. This inability to form biofilm was due to an elevated level of *SinR* and its mRNA. Evidence was given that *YmcA*, *YaaT* and *YlbF* interact with RNase Y which destabilize *sinR* mRNA.

Apart from the role of *SinI*, *SlrR* and *SlrA* are also involved in regulating the DNA binding properties of *SinR*. *SlrR* and *SlrA* are homologs of the *SinR* repressor and *SinI* anti-repressor, respectively (Kobayashi 2008). In the presence of sufficient *SinI*, the gene *slrR* is derepressed as a result of *SinR* inactivation and *SlrR* is produced as a consequence. *SlrR*, once produced, inhibits *SinR* (through titration of *SinR*) and indirectly derepresses its own gene (*slrR*) and matrix operons. The other consequence of *SlrR* synthesis is the formation of the *SlrR*•*SinR* heterocomplex which results in the repression of autolysin and motility genes (σ^D -dependent genes) including the *hag* gene. When *SlrR* binds to *SinR* and a *SinR*•*SlrR* complex is formed, it inhibits the ability of *SinR* to bind to the *slrR* promoter and it can no longer repress biofilm matrix operons. *SinR*•*slrR*•*SlrR* interaction is governed by a self-reinforcing double-negative feedback loop involving protein–protein and protein–DNA interactions (Chai et al. 2010). *SlrR*, alone, as well as in the complex (*SinR*•*SlrR*) regulates motility by acting as a repressor *via* direct interaction with the *hag*, *lytABC* operon, and *lytF* genes (Chai et al. 2010) (Figure 7b). *SinR* is considered to be a master regulator for bistable gene expression. On one side, it represses genes involved in biofilm matrix production and on the other it represses the motility genes, including the *hag* gene (Chai et al. 2009) by forming a complex with *SlrR*, as shown in Figure 7b. The interaction between *SinR* and *SlrA* is established *via* the *YwcC*/*SlrA* pathway; *YwcC* inactivates the *slrA* gene of *SlrA*. *SlrA* is a *SinI* homolog that binds with *SinR* and inactivates it. *B. subtilis* cells during logarithmic growth adapted to one specific way of life: motility (planktonic) or the biofilm mode. One portion of the population chooses to remain in motile state (small motile cells) and the other cells (long cells) form biofilms. Diethmaier et al. (2011) demonstrated the role of a novel factor *Ymdb* in

regulating this switch by affecting both the σ^D and *SinR* regulons, as shown in Figure 7b. It was proposed that *Ymdb* and *SlrR* are a part of a signaling network, which antagonistically controls the expression of motility and biofilm genes. *Ymdb* acts upstream of *SlrR* and thus controls the activity of *SlrR* in response to an input signal of unknown nature. *SlrR* represses motility gene expression and produces active *SinR* to repress biofilm formation. Thus *Ymdb* plays a key role in deciding the cells' way of life during the logarithmic growth stage, when one portion of the population chooses to remain in a motile state (small motile cells), and the other cells elongate and form biofilms. Lei et al. (2013) reported another protein (Veg) that controls the production of matrix in *B. subtilis* by negatively regulating *SinR* activity irrespective of the role of *SinI*, *SlrR* and *SlrA* (Figure 7b).

DegU is another important response regulator in *B. subtilis*. It is a part of the *DegS*-*DegU* two-component signaling system which plays a key role in regulating biofilm formation. *DegU* is phosphorylated by its cognate histidine kinase *DegS*. The *bslA* gene is the main gene that is activated by the intermediate level of *DegU* in its phosphorylated state (*DegU-P*). However, it inhibits biofilm formation at an elevated level (Verhamme et al. 2007). Recently, it has been hypothesized that *DegU-P* at a high level inhibits transcription from the *epsA* and *tapA* promoter regions by reducing the proportion of cells that produce EPS and TasA amyloid fibers and also by controlling the frequency with which cells activate transcription of the genes required for the production of biofilm matrix (Marlow et al. 2014). Recently, it was reported that the *degU* expression is regulated by *SinR*/*SlrR*, which acts negatively and positively, respectively, for the expression of *degU* (Ogura et al. 2014). Figure 7 shows all the pathways involved in mediating the motile–sessile switch in *B. subtilis*.

Concluding remarks and future outlook

Despite hundreds of studies exploring the biofilm mode of life, it still remains the least understood feature of bacterial life, owing to the complex regulatory networks which govern the biofilm mode in a unique way in different bacteria. All transcriptional regulators involved in regulating motility and biofilm matrix production, including the production of e-DNA through cells lysis, have a role in biofilm formation. The c-di-GMP-dependent, signaling pathways control biofilm formation by regulating motility and their role has been acknowledged in many bacterial species. However, the role of c-di-AMP in biofilm formation is not clear yet and it requires more research to explore its mechanistic role and transduction of c-di-AMP signaling in bacteria. Some results, as mentioned in the discussion,

are contradictory and need further work. TA systems have roles in bacterial physiology including biofilm formation mainly through regulating motility and controlling cell lysis.

It is predicted that in the future the role of the novel transcriptional regulator BolA in biofilm formation will be further explored in many Gram-negative species. Most studies aiming to explore the mechanism of biofilm formation are limited to a few model microorganisms of medical significance. A significant gap in biofilm studies is the lack of research on biofilm formation mechanism in many industrially important bacterial species, eg *G. stearothermophilus*, *A. flavithermus* and *Bacillus licheniformis*.

Despite a plethora of studies aiming to explore biofilm forming mechanisms in single species biofilms, there is still a gap between understanding bacterial biofilm forming mechanisms in simple biofilms formed by single controllable bacterial species, and multispecies biofilm communities, which are more significant and prevalent in nature. Current knowledge on multispecies biofilms is very limited and thus there is a dire need for a change in focus from the single-cell level to the community level, and for the exploration of new functions, mechanisms and adaptations of individual organisms in multispecies biofilms.

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