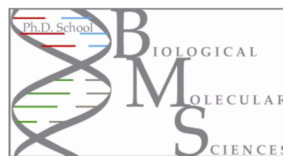
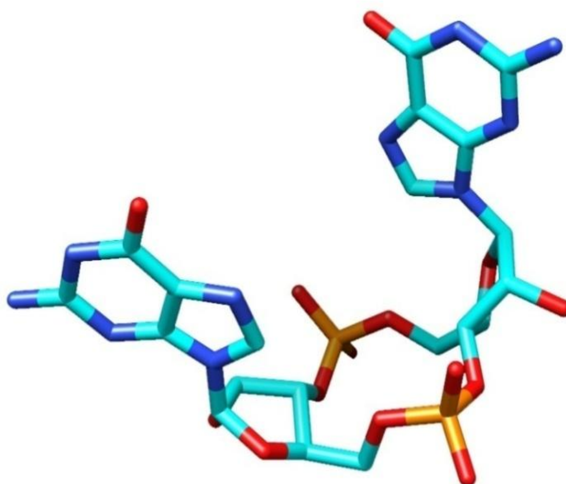


UNIVERSITÀ DEGLI STUDI DI MILANO

Scuola di Dottorato in Scienze Biologiche e Molecolari

XXIV Ciclo

Regulation of bacterial adhesion factors by the signal molecule c-di-GMP: specific effects at gene expression levels and search for novel inhibitors



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Cover image: chemical structure of bacterial second messenger cyclic-di-GMP. Image courtesy of Samuele Agostinelli, Marche Polytechnic University, Ancona Italy.

"Messieurs, c'est les microbes qui auront le dernier mot."

(Gentlemen, it is the microbes who will have the last word.)

Louis Pasetur

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ABSTRACT

Bacteria are able to switch between a single cell (planktonic) lifestyle and a biofilm (community) lifestyle. In pathogenic bacteria, growth as biofilm protects bacterial cells against the host immune system and increases tolerance to antibiotic treatment, thus resulting in chronic infections. The bacterial second messenger cyclic-di-GMP (c-di-GMP) plays a pivotal role in biofilm formation, by promoting production of adhesion factors such as extracellular polysaccharides (EPS). Two classes of enzymes are involved in c-di-GMP metabolism: diguanylate cyclases (DGCs), which synthesize c-di-GMP, and phosphodiesterases (PDEs) that hydrolyze the signal molecule. Usually, a high intracellular c-di-GMP concentration correlates with EPS production and biofilm formation. The enzymes involved in c-di-GMP metabolism are widely conserved in Bacteria, but they are not present in upper eukaryotes. Thus, the proteins involved in c-di-GMP metabolism are a very interesting target for antimicrobial compounds with anti-biofilm activity.

In first part of my thesis I developed a screening system for specific inhibitors of DCGs based on a set of microbiological assays that rely on detection of c-di-GMP-dependent EPS production using specific dyes such as Congo Red. Intracellular c-di-GMP levels can then be measured directly by HPLC determination. I tested over 1,000 chemical compounds in my screening system: I found that azathioprine and sulfathiazole two antimetabolites able to inhibit nucleotide biosynthesis impair c-di-GMP production. My results confirm previous literature data showing that perturbation in intracellular nucleotide pools negatively affect biofilm formation in Gram negative bacteria.

In second part of this thesis I discussed the role of *yddV-dos* operon which encodes a DGC and a PDE acting as a protein complex. Both YddV and Dos proteins affect the production of the main adhesion factors of *Escherichia coli*: curli and the EPS poly-*N*-acetylglucosamine (PNAG). In particular, the YddV-Dos complex regulates transcription of the *csgBAC* operon, which encodes curli structural subunits while not affecting the expression of the regulatory operon *csgDEFG*. In addition we showed that YddV stimulating the transcription of PNAG biosynthetic operon *pgaABCD* affects PNAG-mediated biofilm formation. Thus, the *yddV-dos* operon constitutes a main regulatory element in adhesion factors production.

Finally, I was able to show that PNAG production is controlled by polynucleotide phosphorylase (PNPase) at post transcriptional level. My results demonstrate the integration of signal molecules and regulatory protein in adhesion factor production, underling the complexity of biofilm regulation in *E. coli*

CHAPTER I

INTRODUCTION

1.1 BIOFILMS

In natural environments, microorganisms are often organized in multicellular community growing on surfaces, rather than as free swimming organisms (single-cell behaviour) (Karatan and Watnick 2009). In such communities, called biofilms, microbial cells are embedded in a matrix mainly composed by extracellular polysaccharides (EPSs), proteins and extracellular DNA (eDNA; O'Toole *et al.* 2000; Whitchurch *et al.* 2002; Kolter and Greenberg 2006). This matrix (and more in general the biofilm organization) confers tolerance to antibiotics and protects bacterial cells against environmental and physiological stresses and/or the host immune system (Ryder *et al.* 2007). The biofilm mode of growth differs significantly from the planktonic state (Costerton *et al.* 1995) and the transition from planktonic cells to a biofilm organization is promoted by the expression of a large number of genes encoding cell aggregation and adhesion factors (Costerton *et al.* 1995; Schembri *et al.* 2003a).

The tightly associated cells constituting a bacterial biofilm are able to coordinate their physiological and metabolic state, thus almost resembling the subdivision of functions typical of multicellular organisms (Costerton *et al.* 1995; Shapiro 1998; Caldwell 2002).

Biofilms can have a tremendous impact on human activities. Bacterial contaminations can hamper industrial processes. (Dourou *et al.* 2011; Torres *et al.* 2011) and bacteria adhering to metal surfaces can promote their corrosion leading to substantial economic damages (Costerton *et al.* 1995). Biofilms removal is carried out using either biocides or mechanical methods (*e.g.* grinding, wash-out with high-pressure water), but their complete and efficient removal is often difficult (Bruellhoff *et al.* 2010); for example Kim and colleagues demonstrated that even treatments with ozone are unable to remove biofilm from bean sprouts (Kim *et al.* 2003).

Also the economical activities linked to the medical market are threatened by the presence of biofilms: bacterial adhesion to medical devices (from urinary catheters to contact lens) usually compromises not only the correct functionality of medical device but also the human health (Donlan 2011). Despite extensive efforts, no antimicrobial drug has yet been found that completely eradicates adherent microbial populations (Cos *et al.* 2010), thus, the search for compounds with a specific anti-biofilm action is a very important research topic in applied microbiology and biotechnology (Klemm *et al.* 2010; Rändler *et al.* 2010; Sato *et al.* 2011).

1.1.1 ROLE OF BIOFILMS IN INFECTIONS

Biofilm and planktonic cells differ significantly in their physiology, gene expression pattern, and even morphology. Bacteria growing in biofilms are less sensitive to treatments with antimicrobial agents compared to planktonic cells (Costerton *et al.* 1995; Ceri *et al.* 2001; Mah *et al.* 2003; Martinez and Rojo 2011). Two hypotheses have been formulated to explain the reduced susceptibility to antibiotics by biofilms. The first hypothesis, which could be termed penetration limitation, suggests that only the surface layers of a biofilm are exposed to a lethal dose of the antibiotic due to a reaction-diffusion barrier that limits transport of the antibiotic into the biofilm (Hoyle *et al.* 1992; Kumon *et al.* 1994; Stewart 1994 Anderl *et al.* 2000). The second hypothesis for reduced biofilm susceptibility, which could be termed physiological limitation, proposes that some microorganisms within the biofilm exist in a physiological state intrinsically less sensitive to antibiotic action (Costerton *et al.* 1987; Brown and Gilbert 1993).

A wide variety of medical devices, such as catheters or prostheses, are readily colonized by bacterial biofilms, thus becoming a reservoir of pathogenic bacteria and the starting point for serious human diseases and infections (Donlan 2011). The total amount of death that can be attributed to infections associated to medical devices is worldwide approximately 160,000 per years (WHO estimates). Bone (osteomyelitis, caused prevalently by *Streptococci* and other Gram positive bacteria) and urinary tract (cystitis and urethretis, caused mainly by enterobacteria such as enteropathogenic *Escherichia coli*) infections are mainly due to biofilms, and show remarkable resistance to antibiotic treatment (Trautner *et al.* 2005; Simões 2011). This resistance results in establishment of chronic bacterial infections (Hoyle and Costerton 1991; Finlay and Falkow 1997). Moreover gene transfer is enhanced within biofilm (Ghigo 2001; Li *et al* 2001; Molin and Tolker-Nielsen 2003), thus providing for quick and efficient transfer of antibiotic resistance genes, and making the eradication of biofilm-borne infections difficult to eradicate.

1.1.2 BIOFILM DEVELOPMENT

Transition from the planktonic mode of growth to a more complex structure such as biofilm occurs as a sequential development process (Figure 1.1; Ghigo 2001; Stoodley *et al.* 2002; Reisner *et al.* 2003).

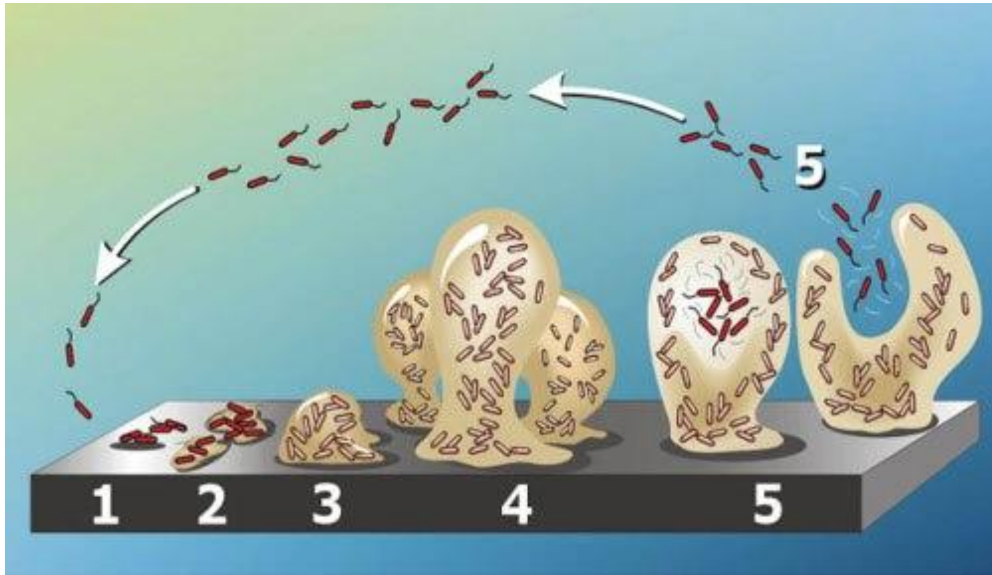


Figure 1.1 Stages of biofilm development. Schematic representation of biofilm development: initial attachment (1), irreversible attachment (2), maturation (3 and 4) and finally dispersal (5). Image from http://2011.igem.org/Team:Glasgow/Biofilm and re-adapted form Monroe 2007.

The process of adhesion to a surface, *i.e.* the first step of biofilm formation, is mostly controlled by physico-chemical properties such as Van der Waals interaction, electrical charge and hydrophobicity of both bacterial cells and surfaces; often bacteria have to overcome electric charge repulsion in order to attach to a surface (Figure 1.1 stage 1; van Loosdrecht *et al.* 1990; Jucker *et al.* 1996). Upon adhesion bacteria might sense contacts with the surface and induce specific gene expression, leading to further development of the biofilm (Davies *et al.* 1993; Sauer and Camper 2001). In the presence of environmental conditions allowing bacterial growth, adherent cells can divide and form an attached monolayer known as a microcolony (Figure 1.1 stage 2). Establishment of stronger cell-cell contacts allows the microcolony to differentiate into a mature biofilm whose three-dimensional structure is determined by the extracellular polymeric substances in which the biofilm is encased (Figure 1.1 stages 3 and 4). Extracellular polymeric substances are mainly constituted by different types of exopolysaccharides (EPS), extracellular proteins and enzymes, and even DNA (Lawrence *et al.* 1991; Whitchurch *et al.* 2002; Karatan and Watnick 2009); in addition, bacterial outer membrane vesicles, flagella, phages, pili, host matrix material, and debris from lysed cell can also be present (Hunter and Beveridge 2005). This extracellular polymeric substances matrix provides structural support to the biofilm, similar to an exoskeleton (Ghigo 2003). Biofilm maturation is characterized by the growth of

surface-attached microcolonies that progress to a mature architecture with increased synthesis of EPS, leading to a complex architecture that includes channels, and pores (Bridier *et al.* 2010). After biofilm maturation, the amount of EPS in the matrix appear to decrease, perhaps due to metabolic changes, with subsequent detachment (Figure 1.1; Stage 5) of clumps and individual cells. These detachment events can take place through mechanic breakage of biofilms, especially when exposed to high flow. However, it was also observed that biofilm cells can induce the production of EPS-degrading enzymes, thus promoting their release from the biofilm (Nijland *et al.* 2010; Abee *et al.* 2011).

1.2 DETERMINANTS IN BIOFILM FORMATION

Transition from planktonic (free-living) cells to the biofilm mode of growth implies substantial modifications regarding the cell morphology and biochemistry (Pratt and Kolter 1999; Schembri *et al.* 2003a). Several features taking part in biofilm formation have been identified, most of which are cell surface-exposed or extracellular structures directly involved in attachment to surfaces and in cell aggregation.

Cell surface factors allowing for initial interaction with surfaces and structure formation include extracellular polysaccharides (Jackson *et al.* 2004; Ryder *et al.* 2007; Karatan and Watnick 2009; Byrd *et al.* 2010), pili (Klausen *et al.* 2003), flagella (O'Toole and Kolter 1998, Klausen *et al.* 2003) and proteins (Monds *et al.* 2007; Newell *et al.* 2009; Borlee *et al.* 2010). These factors are commonly categorized as biofilm matrix components. Interestingly, in recent years, the predominance of nucleic acids among biofilm matrix has lead to the investigation of the importance of DNA in stabilizing the biofilm matrix (Whitchurch *et al.* 2002; Allesen-Holm *et al.* 2006; Yang *et al.* 2007). In the following sections I will provide you an overview about these components.

1.2.1 EXOPOLYSACCHARIDES (EPSs)

Extracellular polysaccharides are the main component of the biofilm matrix (Ryder *et al.* 2007) and play a key role in shaping and providing structural support to the biofilm (Sutherland 2001). These polymers are very diverse and are often involved in the establishment of productive cell to cell contacts that contribute to the formation of biofilms at liquid–solid boundaries, pellicles at air–liquid interfaces, cell aggregates and clumps in liquid cultures, and wrinkled colony morphology on agar plates. The structural role and the regulation of production of these exopolysaccharides are now actively being investigated in

different bacteria (Kirillina *et al.* 2004; Simm *et al.* 2005; Ryder *et al.* 2007). For instance, in *Pseudomonas aeruginosa* alginate is an important matrix molecule for biofilm formation by providing structural stability (Hentzer *et al.* 2001; Nivens *et al.* 2001). Specifically, alginate is a high molecular weight acetylated polymer made up of nonrepetitive monomers of β(1→4) linked L-guluronic and D-mannuronic acids (Figure 1.2A; Evans and Linker 1973). In mucoid strains, alginate is the predominant extracellular polysaccharide of the matrix (Hentzer *et al*. 2001). In addition to alginate, the capsular polysaccharide levan is produced by a subset of *Pseudomonads*, notably by the phytopathogen *Pseudomonas syringae* (Osman *et al.* 1986). Levan is a high molecular mass β-2,6 polyfructan with extensive branching through β(2→6) linkages (Figure 1.2B). Levan is produced exclusively from sucrose through an extracellular levansucrase (Li and Ullrich 2001). *P. aeruginosa* produces at least two other polysaccharides that can be important in biofilm development: PEL and PSL polysaccharides (Colvin *et al.* 2011a). The *pel* locus contains seven genes encoding functions involved in the synthesis and export of an uncharacterized polysaccharide (Colvin *et al*. 2011a). The loss of biofilm formation is specifically attributed to the capability of PEL to initiate and maintain cell–cell interactions (Colvin *et al*. 2011b). The *p*olysaccharide *s*ynthesis *l*ocus (*psl*) contains 12 genes, 11 of which are necessary for PSL synthesis and export (Byrd *et al*. 2009). Recently, the structure of PSL was identified as repeating units of a neutral, branched pentasaccharide consisting of D-glucose, D-mannose and L-rhamnose monosaccharides (Figure 1.2C; Byrd *et al*. 2009). Cellulose is an extracellular matrix component originally identified as an additional determinant for biofilm formation in enterobacteria (Zogaj *et al.* 2003); both medical and environmental isolates of *Escherichia* and *Salmonella* displaying the rough, dry and rugose (rdar) phenotype are capable of producing cellulose-based matrix and robust biofilms which colonize the air-liquid interface of static liquid microcosms (Römling 2005). In *Salmonella* strains, a mutant in cellulose production retains some capability to form cell aggregates, but not a confluent biofilm (Jonas *et al.* 2007). Spiers and colleagues isolated mutants of *Pseudomonas fluorescens* SBW25 that produce biofilms similar to those of the rdar mutants of *Escherichia* and *Salmonella*; in particular in some mutants mutant known as the Wrinkly Spreaders (WS), has been observed an overexpression of partially acetylated cellulose (Spiers *et al.* 2002; Koza *et al.* 2009). Moreover cellulose has been identified as the matrix component in biofilms produced by different environmental *Pseudomonas* isolate (Ude *et al.* 2006). As shown in Figure 1.2D cellulose is a polysaccharide consisting of a linear chain of several hundred to over ten thousand β(1→4) linked D-glucose units.

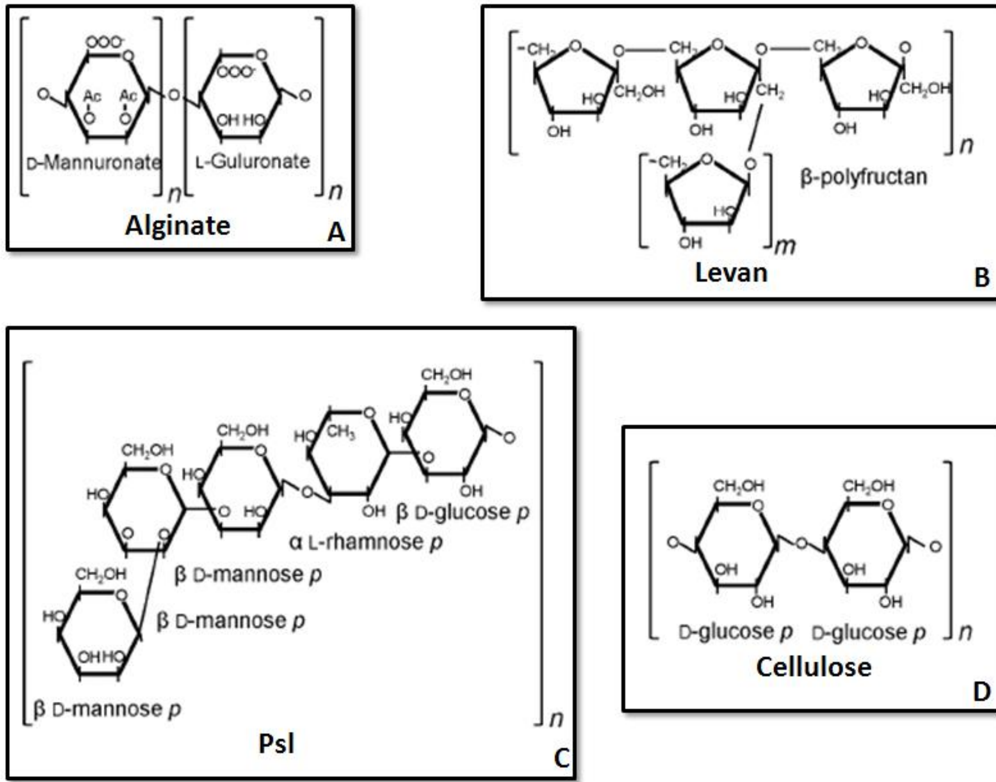


Figure 1.2. Different chemical structures of exopolysaccharides.

In *E. coli*, colanic acid (CA) is induced after attachment to a solid surface (Davies and Geesey 1995). In agreement with this observation, CA synthesis does not appear critical for initial colonization but rather for the formation of the complex three-dimensional structure of biofilms (Danese *et al.* 2000; Prigent-Combaret *et al*. 2000). It has been demonstrated that CA plays an important role during biofilm formation by *Salmonella enterica* serovar *typhimurium* on HEp-2 cells (cells of epidermoid carcinoma) and chicken intestinal epithelium (Ledeboer and Jones 2005). CA biosynthesis is extremely complex: in *E. coli* it involves 19 genes, clustered in the *wca* locus (Stevenson *et al.* 1996). Interestingly, although CA synthesis is widely present in the *Enterobacteriaceae*, the genes involved in its biosynthesis are not highly conserved (Stevenson *et al.* 1996). Despite CA critical role in biofilm development CA overproduction can result in biofilm inhibition in *E. coli* BW25113 strains (Zhang *et al.* 2008).

Biofilm formation of *Staphylococcus epidermidis* and *Staphylococcus aureus* is mediated by the polysaccharide intercellular adhesin (PIA) encoded by the *icaABCD* operon (Fluckiger *et*

al. 2005). PIA is an important factor in colonization of medical devices and in cell-cell adhesion (Heilmann *et al.* 1996; McKenney *et al.* 1998; Conlon *et al.* 2002). PIA production actively protects the bacteria against major components of the human immune system, such as leukocytes and antibacterial peptides (Vuong *et al.* 2004). In particular *icaA* is a glycosyltransferase which catalyses the assembly of large polymers of *N*-acetylglucosamine residues (Heilmann *et al.* 1996; Gerke *et al.* 1998). Also other *Staphylococcus* species, such *S. caprae*, were found to form biofilms by *icaABCD*-dependent PIA production (Cramton *et al.* 1999; Allignet *et al.* 2001). It appears that *ica*-like genes encode proteins responsible for the production of extracellular polymeric substance in a widely distributed group of bacteria. Homologous genes responsible for biofilm formation are found in *Yersinia pestis* (*hms* locus) and also *E. coli* (*pga* locus) (see chapter III; Darby *et al*. 2002; Joshua *et al.* 2003; Wang *et al.* 2004a).

1.2.2 LIPOPOLYSACCHARIDE (LPS)

The lipopolysaccharide, also known as lipoglycan, is the main component of the outer membrane of Gram negative bacteria, and it consists of three subunits: lipid A, core oligosaccharide and O-specific antigen or O-side chain. LPS has been shown to be involved in interactions, either attraction or repulsion, of bacteria with solid surfaces, such as glass beads or Teflon (Jucker *et al.* 1996). In *E. coli* W3100, knock-out mutations in *rfaG*, *rfaP* and *galU* genes, which are involved in LPS core biosynthesis, lead to a decreased ability to adhere to polystyrene surfaces, and *galU* and *galE* mutants of *Vibrio cholerae* are not able to form biofilm (Nesper *et al.* 2001). However, the loss of the adhesion seems to be caused by the alteration of type I fimbriae and/or flagella, associated with these mutations, rather than a direct role of LPS in cell-surface interactions (Genevaux *et al.* 1999). In *E. coli* W3100 grown under anoxic conditions, the ability to adhere to hydrophilic surfaces was negatively affected by higher production of LPS, while inactivation of *waaQ*, which is part of the LPS core biosynthetic operon, stimulated adhesion both under aerobic and anoxic conditions, suggesting a negative role of LPS in adhesion to sand (Landini and Zehnder 2002). In contrast, several strains defective in LPS synthesis, such as *Klebsiella pneumonia*, *Proteus mirabilis* and *Serratia marascens*, were found to have reduced ability to adhere to uroepithelial cells, as well as to form biofilms (Izquierdo *et al.* 2002). Thus LPS can contribute in different ways to adhesion properties of a cell, by either bridging the gap between cell and surface or inhibiting attachment through steric hindrance of such a bridging (van Loosdrecht *et al*. 1990; Rijnaarts *et al.* 1993).

1.2.3 PILI AND FIMBRIAE

Pili and fimbriae are extracellular structures constituted by proteins. Type I fimbriae are short and numerous, and are encoded by the *fim* genes and expressed in most *E. coli* and *Salmonella* strains (Dwyer *et al.* 2011; Puorger *et al.* 2011 Tchesnokova *et al.* 2011). Type I fimbrae play a key role in the colonization of various host tissues as well as in biofilm formation on abiotic surfaces and in autoaggregation (Pratt and Kolter 1998; Schembri and Klemm 2001; Boddicker *et al.* 2002). Fimbriae are dispensable for the establishment of initial cell-surface contacts, but appear to be essential for the stabilization of cell-cell contacts in later steps of biofilm formation. Deletion of entire *fim* cluster results in increased expression of Antigen 43 (Ag43), a surface protein, encoded by the *flu* gene (Schembri *et al.*, 2003b). Ag43 mediates cell-cell or cell-surface contacts and promotes biofilm formation in glucose minimal medium in *E. coli* (Danese *et al.* 2000). In contrast to other surface structures such as fimbriae, Ag43 adhesin is directly anchored to the outer membrane, thus resulting in a more intimate cell-cell contact than in other cellular interactions. Another kind of fimbriae, called autoaggregative adherence fimbriae (AAF), is a determinant for biofilm formation by enteroaggregative *E. coli* (Sheikh *et al.* 2001).

Pili are generally longer than fimbriae; they can serve as specific receptors for bacteriophages and are involved in the process of conjugation. *E. coli* cells can establish tight cell-cell contacts through F-pili. Such pili promote horizontal gene transfer of genetic material between donor and recipient cells, transfer that appear to take place with higher frequency in biofilms than in planktonic cells. Type F-pili are encoded by natural conjugative plasmids, which thus direct the expression of biofilm factors as part of a coordinated strategy aimed to their propagation (Ghigo, 2001). In *Pseudomonas* type IV pili, involved in surface-associated twitching motility, appear to be necessary for microcolony formation: indeed, mutants unable to express type IV pili cannot progress beyond the initial adhesion step and form microcolonies (O'Toole and Kolter 1998). Another study found that type IV pili are induced in biofilm cells, whereas planktonic cells lack these structures, suggesting a role of twitching motility within the biofilm (Sauer and Camper 2001). Biofilm-dependent expression of type IV pili is only one of several examples of switching the production of different kinds of pili according to the environmental cues and physiological conditions. For instance, *V. cholerae* expresses TCP (toxin-coregulated pilus, belonging to the type IV pili group) in the host intestine, where it serves as an essential colonization

factor, while attachment to abiotic surfaces such as borosilicate is mediated by the mannose-sensitive hemagglutin (MSHA) pilus (Watnick *et al*. 1999).

1.2.4 OUTER MEMBRANE PROTEINS (OMPs)

Proteins located in the outer membrane of Gram negative bacteria are often involved in cell-surface attachment. Actually OMPs probably affect surface structures rather than play an active role in cell-surface interaction. Type 1 fimbriae-mediated surface contact leads to distinct changes in the outer membrane protein composition, including reductions in the levels of many outer membrane proteins (Otto *et al.* 2001). These alterations imply that a change in the cell surface takes place immediately in response to attachment.
Inactivation of *ompX* leads to enhanced fimbriation, significantly increased surface attachment and impairment of motility. Moreover, inactivation of *ompX* results in an approximately threefold increase in the production of EPS (Otto and Hermansson 2004). Thus, OmpX likely affects regulation and/or cell localization of different surface structures.

1.2.5 EXTRACELLULAR DNA (eDNA)

Extracellular DNA (eDNA) is an important component of the biofilm matrix. It is released by autolysis and acts as an adhesive (Vilain *et al.* 2009) and strengthens biofilm (Whitchurch *et al.* 2002). It was demonstrated that *P. aeruginosa* biofilms in early development stage were strongly inhibited by treatment with DNaseI, although cell viability was not affected. In contrast, mature biofilms were not sensitive to treatment with DNase I, suggesting that eDNA is important only at the early stages of biofilm development (Whitchurch *et al.* 2002). In addition to the structural role of eDNA, intracellular levels of cytidine influence extracellular polysaccharides biosynthesis and surface attachment in *V. cholerae*, thus suggesting that nucleosides might act as signals for biofilm formation (Haugo and Watnick 2002). Finally, *Streptococcus gordonii* mutants defective in competence genes were found attenuated in biofilm formation (Loo *et al.* 2000; Yoshida and Kuramitsu 2002). Such competence mutants are also defective in autolysis suggesting that not enough eDNA might be present to initiate biofilm formation in these strains.

1.3 REGULATION OF BIOFILM FORMATION

Gene expression regulation of biofilm determinants often requires a combination of different environmental signals, which can modulate the activity of complex regulatory networks of

both specific and global regulators. Interestingly, despite the striking physiological change represented by the transition to biofilm, only a few biofilm-specific genes and very little in terms of biofilm-dedicated pathways have been revealed thus far (Ghigo 2003; Landini 2009). Adhesion and/or aggregative cellular factors can be part of environmental stress regulons (*i.e.* nutritional or oxidative stress), which can directly affect transition from single cells to biofilm, biofilm maintenance and even dispersal. In this section I will review the common mechanisms that regulate biofilm formation; I will then focus my attention on c-di-GMP, a bacterial second messenger that plays a pivotal role in biofilm formation (Hengge 2009; Schrimer and Jenal 2009). c-di-GMP is the subject of my experimental work.

1.3.1 TRANSCRIPTIONAL REGULATION RESPONDING TO ENVIRONMENTAL SIGNALS

Bacterial gene expression is mainly regulated at the transcriptional level in response to external stimuli or stresses. Many transcription factors, either global or specific, can influence biofilm formation. For instance, expression of curli fibers, the main adhesion factor in *E. coli* strains, is regulated by low temperature, low osmolarity conditions and by nutrient starvation (Olsen *et al.* 1993; Gerstel and Römling 2001). Temperature regulation also plays a role in the expression of outer biofilm determinants, such as the *Y. pestis hms* genes, responsible for PIA production: the transcription of these genes is repressed upon a temperature shift from 26°C to 34°C (Perry *et al.* 2004). The presence of a specific nutrient can trigger opposite effects in different bacteria: for instance, biofilm formation by *E. coli* K12, *S. aureus* and *Streptococcus mutans* is repressed by the presence of glucose (Regassa *et al.* 1992; Jackson *et al.* 2002; Shemesh *et al.* 2007), which, in contrast, promotes biofilm formation of enteroaggregative *E. coli* (Sheikh *et al.* 2001) and of *Salmonella enteriditis* (Bonafonte *et al.* 2000). Glucose-mediated regulation of biofilm formation appears to take place at two different levels: through the cAMP/CAP regulon (transcriptional regulation) and by the CsrA protein (post-transcriptional regulation). Presence or absence of oxygen is another signal with high influence on biofilm formation: indeed during *P. aeruginosa* chronic infection of the cystic fibrosis lung, oxygen-limiting conditions seems to contribute to persistent infection; oxygen limitation increases antibiotic tolerance, and induces biofilm formation and alginate biosynthesis (Schobert and Tielen 2010). In contrast, growth in oxygen-limited conditions results in a sharp decrease in *E. coli* adhesion to hydrophilic substrates (Landini and Zehnder 2002).

Influence of environmental cues can be mediated by two-component regulatory systems (TCRS) that can sense the changes in the environmental conditions and trigger a specific cellular response. TCRS are constituted by a sensor protein, usually found in the membrane, and by a regulatory protein, able to bind specific sequences on the DNA (Mikkelsen *et al.* 2011). Transcription regulation is triggered by chemical modification of an inactive regulatory protein (usually by phosphorylation) carried out by the sensor protein. Several TCRS are directly involved in biofilm formation; an example is the *cpxA*/*cpxR* system involved in control of curli biosynthesis. It is composed by CpxA, a sensor kinase and phosphatase, and CpxR, a response regulator (Danese and Silhavy 1997). These genes are induced by general stress conditions in the periplasmic compartment resulting in protein denaturation. The *cpx* system is involved in surface sensing and promoting adhesion (Jubelin *et al.* 2005). A CpxR mutant strain forms less stable abiotic surface-cell interactions in comparison to the wild type strain (Otto and Silhavy 2002). Consistent with this, when *E. coli* cells interact with a hydrophobic surface, the Cpx pathway is activated (Otto and Silhavy 2002). In addition to stable cell surface interactions being regulated by sensing contact with a surface, these interactions can also be regulated by environmental conditions, specifically increased osmolarity. The EnvZ/OmpR signaling system, appears to have a role in promoting stable cell–surface interactions in response to increased osmolarity (Otto and Silhavy 2002). A strain of *E. coli* with a mutation in the OmpR protein (*OmpR234* allele) responsible for hyperactivation of the curli-encoding operons, leads to increased adhesion (Vidal *et al.* 1998; Prigent-Combaret *et al.* 2001). The EnvZ/OmpR signalling system is activated to generate phosphorylated OmpR under conditions of increasing osmolarity (Pratt and Silhavy 1995), suggesting that increased osmolarity would stimulate stable cell–surface interactions. Several TCRS such as PhoQP influence expression of EPS biosynthesis, for instance colanic acid, thus affecting biofilm formation, in response to external concentrations of divalent cations such as zinc and to glucose availability (Hagiwara *et al.* 2003).

1.3.2 INTRACELLULAR SIGNAL MOLECULES

Products of amino acids degradation may function as intracellular signal molecules involved in adhesion. The amino acid tryptophan can be hydrolyzed by the enzyme tryptophanase to form indole and pyruvate: while the latter is further degraded in the TCA cycle, indole accumulates in the cell where it can have a stimulatory effect on biofilm formation. A study of the role of tryptophanase and indole in biofilm formation by a number of clinical isolates of *E. coli*, *Klebsiella oxytoca*, *Providencia stuartii*, *Citrobacter koseri*, *Morganella morganii*,

and *Haemophilus influenzae* showed that the presence of a tryptophanase inhibitor in the culture medium prevented biofilm formation but had no effect on growth (Di Martino *et al.* 2003). In *V. cholerae* transposon insertions in the tryptophanase gene led to a "rugose to smooth" shift in colony morphology, which was reversed by addition of exogenous indole (Mueller *et al* 2007). Therefore it has been proposed that indole can acts as a signal molecule (Wang *et al.* 2001). Genes necessary for indole production have been shown to be induced by addition of *E. coli* stationary phase supernatant (Ren *et al.* 2004), suggesting that they can be induced by mechanisms akin to quorum sensing (see next section).

Polyamines, such as putrescine and norspermidine, are linear organic molecules containing two or more amine groups that are positively charged at neutral pH. They are essential for cell growth, and their intracellular levels are tightly regulated by synthesis, import, export, and interconversion (Tabor and Tabor 1984). Recently, several reports have suggested that polyamines may function as extracellular and/or metabolic signals that modulate biofilm formation. Norspermidine, increases biofilm formation by *V. cholerae* (Karatan *et al.* 2005). *Y. pestis* mutants unable to synthesize putrescine are impaired in biofilm formation. This defect can be rescued in a dose-dependent manner by supplementation of the growth medium with putrescine, suggesting that biofilm formation can activate by both exogenous and endogenous putrescine (Patel *et al.* 2006). Furthermore, norspermidine and putrescine transporters have been implicated in surface-associated growth of *Agrobacterium tumefaciens* and *Pseudomonas putida* (Matthysse *et al.* 1996, Sauer and Camper 2001).

1.3.3 QUORUM SENSING

The differentiation from microcolony to a mature biofilm embedded in an EPS matrix seems to be triggered by both extracellular factors and quorum sensing signals. Quorum sensing (QS), a term introduced by Clay Fuqua and colleagues (Fuqua *et al*. 1994), is an example of cell-to-cell communication and depends on small, diffusible signal molecules called autoinducers (Kaplan and Greenberg 1987). QS is typically involved, in the regulation of genes involved in biofilm maturation and maintenance (Hammer and Bassler 2003; Vuong *et al.* 2003; Ueda and Wood 2009). Indeed, since QS-controlled regulatory pathways are activated at high bacterial cell density, it is not surprising that QS is induced in biofilms, where local cell concentrations can be more than ten fold higher than planktonic cultures. In addition to its role in biofilms, QS can control production of virulence factors in both Gram positive and Gram negative pathogenic bacteria (Kong *et al.* 2006; Xu *et al.* 2006; Hegde *et al.* 2009).

The signals molecules are produced and secreted during bacterial growth. Their concentrations in the environment accumulate as the bacterial population increases, and when it reaches a threshold level (quorum), it induces phenotypic effects by regulating the expression of target genes. Although regulation by QS is highly conserved in bacteria, its molecular mechanisms, as well as the chemical nature of the autoinducers, differ significantly between Gram positive and Gram negative bacteria (Figure 1.3; reviewed in Miller and Bassler 2001).

The best characterized QS mechanism, typical of Gram negative bacteria, involves production and response to small signal molecules belonging to the *N*-acyl-homoserine lactones (AHLs) family (Fuqua *et al.* 1994; Fuqua *et al.* 1996), additional species-specific QS systems make use of other autoinducers, such as quinolonones in *P. aeruginosa* (McKnight *et al.* 2000), or the diffusible signal factor (DSF), a fatty acid (cis-11-methyl-dodecenoic acid) used as signal molecule by the plant pathogen *Xanthomonas campestris* (Barber *et al.* 1997). These density-dependent regulatory systems rely on two proteins, an AHL synthase, usually a member of the LuxI family protein, and an AHL receptor protein belonging to the LuxR family of transcriptional regulators. At low population densities cells produce a basal level of AHL via the activity of an AHL synthase.

When cell density increases, AHL accumulates in the growth medium; on reaching a critical threshold concentration, the AHL molecule binds to its cognate receptor which in turn leads to the induction/repression of AHL-regulated genes (Figure 1.3A; Eberl 1999).

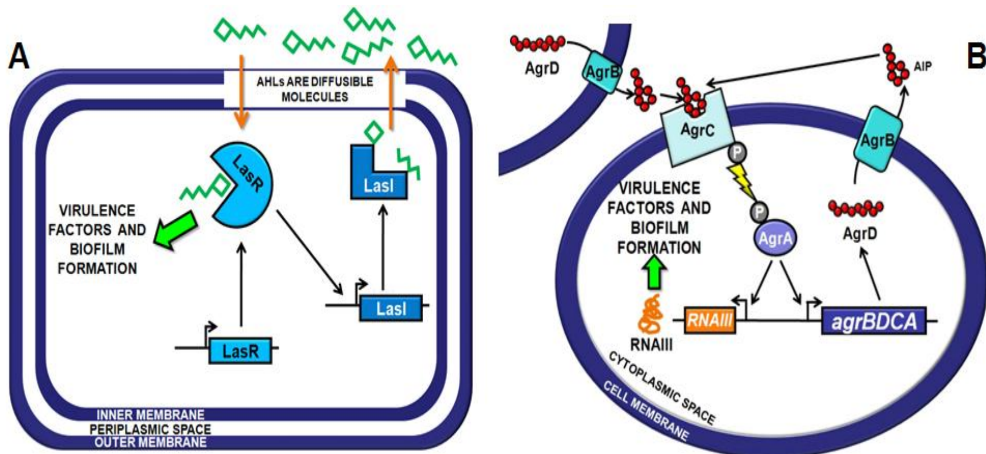


Figure 1.3. Summary of regulatory processes controlling biofilm formation, maintenance, and dispersal in the Gram negative bacterium *P. aeruginosa* (**A**) and in the Gram positive bacterium *S. aureus* (**B**). **A** Acyl-homoserine lactone autoinducers (AHLs; represented by the green diamond and the squiggly line) can diffuse through the cell membranes. AHLs accumulation and binding to the LasR protein trigger activation of biofilm- and virulence-related genes (above in the figure). **B** The AgrD oligopeptide (the QS autoinducer peptide, or AIP) is synthesized as a linear peptide modified and exported by the AgrB protein. Its accumulation leads to interaction with the AgrC sensor protein, which phosphorylates the AgrA response regulator, leading to transcription activation of virulence related genes.

In contrast to Gram negative bacteria, the typical quorum sensing signal molecules in Gram positive bacteria are short peptides (5–50 amino acids), synthesized by ribosomes and often subjected to extensive post-translational modification (Miller and Bassler 2001). Binding of signaling peptides to sensor proteins in the cell membrane triggers a signal transduction cascade, which leads to phosphorylation of a response regulator and triggers QS-dependent gene expression. A model of QS systems in Gram positive bacteria is the *agr* (accessory gene regulation) system of *S. aureus* (Figure 1.3B), where autoinducer-dependent phosphorylation of the AgrA regulator leads to transcription activation of a number of genes, many of which are involved in bacterial virulence (Novick *et al.* 1993; Balaban and Novick 1995).

QS regulates several events in *P. aeruginosa*, such as the production of virulence factors and secondary metabolites, as well as the adaptation and survival in stationary phase. It also affects biofilm, since mutants lacking the autoinducer produce thinner, less structured biofilms which are more susceptible to biocides; however, biofilm formation is not completely impaired (Davies *et al.* 1998). Within the biofilm, quorum sensing-dependent genes are expressed at higher levels in cells near the surface, and expression decreases with the depth of the biofilm (De Kievit *et al.* 2001). Thus, QS is required for the differentiation

of individual cells to a complex multicellular structure and differentiation of the mature biofilm into the typical mushroom-like structure, rather than for the first steps of biofilm formation.

1.3.4 GLOBAL REGULATORS

Several global regulatory proteins are involved in biofilm formation. Many global regulators display low level specificity in DNA binding and regulate transcription of many genes by modifying the architecture of their regulatory regions. H-NS and RpoS, associated with responses to environmental conditions, play a role in modulating biofilm formation. H-NS is a nucleoid-associated protein that can regulate a large number of genes in *E. coli* (approximately 5% of the *E. coli* K-12 genome) (Soutourina *et al.* 1999), including numerous cell envelope components such as type I fimbriae, LPS, and colanic acid, most of them regulated by environmental stimuli including pH, oxygen, temperature, and osmolarity (Dorman and Nì Bhriain 1992; Olsén *et al.* 1998; Hommais *et al.* 2001; Dorman 2004). The H-NS protein directly affects biofilm formation by inhibiting the interactions between σ^{70}-RNA polymerase (the main form of RNA polymerase during the exponential phase of growth) and promoters. However, RNA polymerase associated with σ^{s}, an alternative σ factor mainly active in stationary phase, can bypass H-NS inhibition. This effect mediated by the H-NS protein is called exponential silencing and also takes place at the *csgBA* promoter, thus preventing transcription of the structural units of curli subunits during exponential phase of growth (Arnqvist *et al.* 1994). In *E. coli* strains unable to produce curli, *hns* mutants display better adhesion properties when grown in anaerobic conditions. H-NS inhibition of adhesion is mediated by lower LPS and FliC (flagellin) production, which can act as negative determinants for initial attachment to hydrophilic surfaces (Landini and Zehnder 2002). Thus, H-NS appears to be a negative determinant for biofilm formation.

The alternative σ^{s} subunit of RNA polymerase (also called RpoS protein) is a master regulator of general stress response and it directly regulates adhesion factors such as curli, however its role in biofilm formation is complex and in some cases still controversial. RpoS governs the expression of many genes induced during the stationary phase of growth; in *P. aeruginosa* RpoS expression appears to be related to the QS system through mutual control (Latifi *et al.* 1996; Whiteley *et al.* 2000). Thus, RpoS was thought to play a key role in biofilm formation in many bacterial species. Indeed, *rpoS* mutants of *E. coli* build thinner biofilm when grown in continuous cultures (Adams and McLean 1999). Schembri *et al.*

found that 46% of RpoS-dependent genes are differently expressed in biofilms and that the deletion of *rpoS* lead to an *E. coli* strain incapable of establishing sessile communities (Schembri *et al.* 2003a). In contrast, other investigators reported that expression of RpoS in *P. aeruginosa* is repressed in biofilms, and *rpoS*-deficient mutants not only formed better biofilms than wild type cells, but were more resistant to antimicrobial treatment (Whiteley *et al.* 2000). Consistent with these findings, RpoS seems to negatively influence expression of type I fimbriae in *E. coli*, which can also mediate biofilm formation (Dove *et al.* 1997). Thus it is possible that RpoS can play both a negative and a positive role in biofilm formation.

1.3.5 SMALL RNAs AND BIOFILM REGULATION

In bacteria, more than 150 non-coding small RNAs (sRNAs) have been described (Livny and Waldor 2007). Most bacterial sRNAs affect gene expression regulation, usually at post-transcriptional level and in collaboration with the RNA chaperone Hfq. sRNAs co-interact with specific mRNA targets, thereby modifying the accessibility of the Shine-Dalgarno sequence to the translational machinery and thus altering mRNA stability. A second type of post-transcriptionally active sRNAs interacts with RNA-binding regulatory proteins of the widely conserved RsmA/CsrA family. RsmA (regulator of secondary metabolism) and CsrA (carbon storage regulator) are found in *P. areuginosa* and in *E. coli* respectively, where they act as translational repressors; sRNAs having high affinity for these proteins are therefore able to relieve translational repression by sequestering them (Babitzke and Romeo 2007). Recently, it has been discovered that many of these sRNA are involved in the expression regulation of biofilm formation. For instance, CsrA in addition to being involved in carbon flux regulation, is involved in the control of motility and biofilm formation. CsrA activity is counteracted by CsrB and CsrC sRNAs (Liu *et al.* 1997; Dubey and Babitzke 2005; Suzuki *et al.* 2006) which contain multiple CsrA binding sites that bind and sequester CsrA. Moreover, recently it has been described that the expression of CsgD, the transcriptional activator of curli genes, is in part controlled post-transcriptionally by two redundant sRNAs, OmrA and OmrB (Holmqvist *et al.* 2010).These observations suggest that our understanding of sRNA-dependent regulation of biofilm related genes is still very limited.

1.3.6 c-di-GMP METABOLISM: GGDEF AND EAL PROTEINS

Cyclic di-GMP (bis-(3′-5′)-cyclic di-guanosine monophosphate) is a bacterial second messenger implicated in regulation of diverse processes including developmental transitions, aggregative behavior, adhesion, biofilm formation, and virulence in human pathogens (Hengge 2009; Karatan and Watnick 2009; Schrimer and Jenal 2009). Cyclic di-GMP was originally described in 1987 as an allosteric regulator of cellulose synthesis in *Gluconacetobacter xylinus (*Ross *et al.* 1987). It was subsequently shown that enzymes from *G. xylinus* involved in cyclic di-GMP (c-di-GMP) synthesis and degradation are diguanylate cyclases (DCGs) and phosphodiesterases (PDEs); these enzymes contain the conserved GGDEF and EAL domains respectively (Tal *et al.* 1998; Hengge 2009; Sondermann *et al.* 2012). The GGDEF and EAL nomenclature relates to the conserved amino-acid motifs in these domains which define the proteins catalytic sites (Galperin *et al.* 2001): indeed mutations in the GGDEF and EAL motifs result in loss of enzymatic activity (Chan *et al.* 2004; Kirillina *et al.* 2004; De *et al.* 2008; Bassis and Visick 2010). *In vitro* and *in vivo* studies show that GGDEF domain converts two molecules of GTP to cyclic di-GMP but has no activity with other nucleotides (Figure 1.4; Ryjenkov *et al.* 2005; De *et al.* 2008). Biochemical evidences indicate that the EAL domain hydrolyzes cyclic di-GMP first to generate the linear nucleotide 5′-phosphoguanylyl-(3'-5')-guanosine (pGpG) and then albeit with a much slower kinetic two GMP molecules (Figure 1.4; Christen *et al.* 2005; Schmidt *et al.* 2005; Tamayo *et al.* 2005). Bioinformatic studies suggested that a third domain, HD-GYP, might be involved in cyclic di-GMP hydrolysis (Galperin *et al.* 2001). This was indeed demonstrated by studies showing that HD-GYP proteins are able to hydrolyze c-di-GMP directly in two GMP molecules (Schmidt *et al.* 2005; Dow *et al.* 2006; Hengge 2009; Ryan and Dow 2010)

Tipically DGCs function as homodimer of two GGDEF subunits. The active site (A site) is located at the interface between the two subunits, each binding one molecule of GTP (Chan *et al.* 2004; Christen *et al.* 2005). The activity of DGC is modulated by the binding of c-di-GMP at the allosteric inhibitory site (I site); in general GGDEF domains contain a RxxD I-site motif (Yang *et al.* 2011).

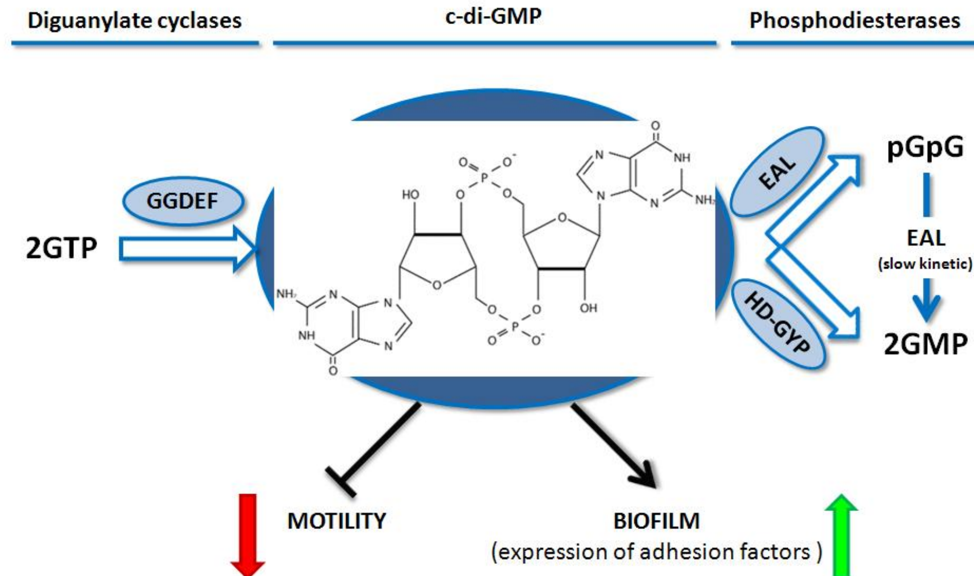


Figure 1.4. c-di-GMP structure, function and metabolism. In cells, bis-(3'-5')-cyclic dimer guanosine monophosphate (c-di-GMP) levels are controlled by duguanylate ciclases (GGDEF) and phosphodiesterases (EAL and HD-GYP) proteins. Low intracellular c-di-GMP concentrations can reduce motility by downregulating flagellar expression or assembly, or interfering with flagellar motor function. High c-di-GMP levels are required for the stimulation of various biofilm-associated functions, such as formation of fimbriae and others adhesions factors. pGpG, 5'-phosphoguanylyl-(3'-5')-guanosine.

Proteins involved in c-di-GMP turnover usually carry only one c-di-GMP related domain and are thus defined GGDEF-only, EAL-only or HD-GYP-only proteins (Seshasayee *et al.* 2010) Sometimes there are also hybrid proteins containing both GGDEF and EAL domains in what has been termed a "biochemical conundrum" (Ryan *et al.* 2006; Seshasayee *et al.* 2010). One possible explanation is that in some cases one of the two domains is not functional (Lacey *et al.* 2010); alternatively proteins can have both activities but they could switch between states in which either their DGC or their PDE activity can prevail (Ryan *et al.* 2006).

Large-scale sequencing of bacterial genomes has revealed that GGDEF and EAL domains are highly abundant and widely distributed, although they are not found in any archaeal genome sequenced thus far (Galperin *et al.* 2001; Römling *et al.* 2005). In January 2012 Pfam database (web url http://pfam.sanger.ac.uk/) reported that GGDEF/EAL domain are absent in human genome and are present only in few eukaryotes like *Ricinus communis* (castor oil plant) *Oryza sativa var. japonica* (Japanese rice) and *Nematostella vectensis* (starlet sea anemone). In addition it is possible to identify 27762 GGDEF sequences and 17210 EAL sequences respectively among 1970 and 1734 bacterial species (Pfam Database

January 2012). In general the number of GGDEF/EAL proteins is larger in Gram negative bacteria than in Gram positive; this may suggest that, Gram positive bacteria do not appear to use this molecule as extensively to regulate cellular behaviors (Table 1.1; Holland *et al.* 2008; Karatan and Watnick 2009)

Species	DGCs	PDEs
Vibrio vulnificus	64	32
Pseudomonas aeruginosa	32	22
Escherichia coli	18	17
Bacillus subtilis	4	3
Staphylococcus aureus	1	1

Table 1.1 Number of diguanylate cyclases (DCGs) and phosphodiesterases (PDEs) in different bacterial species. Source Pfam database http://pfam.sanger.ac.uk/ (January 2012).

Most DGCs or PDEs are modular: in addition to their GGDEF, EAL, or HD-GYP domains, they have a variety of sensory domains (PAS, PAC, GAF, BLUF, HAMP and others) that are likely to receive signals from the environment (Figure 1.7 page 28; Galperin 2004). In particular PAS domains are found in a large number of organisms from bacteria to humans. The name PAS derives from three proteins in which it occurs: **P**er (period circadian protein) **A**rnt (aryl hydrocarbon receptor nuclear translocator protein) **S**im (single-minded protein). PAS domains have important roles as sensory modules for oxygen tension, redox potential or light intensities (Taylor and Zhulin 1999). PAC motifs occur C-terminal to a subset of all known PAS motifs; it is proposed to contribute to the PAS domain fold (Ponting and Aravind 1997). GAF domain found in c**G**MP-specific phosphodiesterases, **a**denylyl cyclases and **F**hlA is also present in phytochromes and in NifA, a transcriptional activator which is required for activation of most *nif* operons which are directly involved in nitrogen fixation (Ho *et al.* 2000). The BLUF domain has been shown to bind FAD in the AppA protein; this one is involved in the repression of photosynthesis genes in response to blue-light. BLUF domain is also found in the DGC YcgF in *E. coli* (Tschowri *et al.* 2009). Finally HAMP domain (present in **H**istidine kinases, **A**denyl cyclases, **M**ethyl-accepting proteins and **P**hosphatases) is found in bacterial sensor and chemotaxis proteins and in eukaryotic histidine kinases (Pham and Parkinson 2011; Mondéjar *et al.* 2012). Physiological and environmental signals are thought to be transduced through an alteration of the enzymatic activity that would result in local or global fluctuations in c-di-GMP levels, which in turn would result in behavioral adjustments (Jenal and Malone 2006; Römling and Amikam 2006;

Ryan *et al*. 2006) In general, high intracellular c-di-GMP concentration stimulates the biosynthesis of adhesins and of exopolysaccharide-based matrix and inhibits various forms of motility: it controls switching between the motile planktonic and sedentary biofilm-associated 'lifestyles' of bacteria (Figure 1.4; Simm *et al.* 2005; Hengge 2009; Boehm *et al*. 2010; Paul *et al*. 2010). Moreover, c-di-GMP controls the virulence of animal and plant pathogens (Tamayo *et al.* 2007; Matilla *et al.* 2011), progression through the cell cycle (Duerig *et al.* 2009), antibiotic production (Fineran *et al.* 2007); and other cellular functions. The ability of a second messenger to have numerous effects on cellular behavior lies in the diversity of c-di-GMP receptors, which act as effector molecules. These receptors monitor the c-di-GMP level in the cell and translate it in to a specific behavioral response (Mills *et al.* 2011).

One method by which c-di-GMP effectors respond to intracellular synthesis or degradation of c-di-GMP is through a c-di-GMP-binding protein domain termed the PilZ domain ("Pills domain"; Figure 1.5; Amikam and Galperin 2006). c-di-GMP allosterically affects activity of many enzymes by binding to a PilZ domain. The cellulose synthesis enzyme of *Salmonella typhimurium*, BcsA, is one example: this enzyme contains a cytoplasmic PilZ domain, which is thought to regulate the enzymatic activity of a periplasmic cellulose synthesis domain, as a consequence of c-di-GMP binding (Zogaj *et al*. 2001). Alginate production by *P. aeruginosa* is also regulated by binding of c-di-GMP to the PilZ domain of the predicted alginate synthesis enzyme Alg44 (Merighi *et al*. 2007). Another mechanism by which effector proteins respond to c-di-GMP levels is through degenerate GGDEF and EAL domains. Proteins that contain GGDEF or EAL domains, which have lost catalytic activity, can bind c-di-GMP at an allosteric c-di-GMP-binding site of a GGDEF domain, or the catalytic site of an EAL domain, respectively. One example of this is the LapD protein from *P. fluorescens,* which contains both GGDEF and EAL domains. The GGDEF and EAL domains are degenerate and show no enzymatic activity *in vitro*. Instead, LapD is a c-di-GMP receptor, binding c-di-GMP to its degenerate EAL domain (Figure 1.5; Newell *et al*. 2009). c-di-GMP binding to LapD activate a complex molecular mechanism which promotes the maintenance on the cell surface of the specific adhesin LapA (Newell *et al*. 2009) Other non-PilZ-, non-GGDEF/EAL-domain proteins have evolved the ability to sense c-di-GMP through the evolution of domains that previously had some other function, such as binding other small nucleic acids. One example of this is *X. campestris* Clp protein, a cyclic-AMP-receptor-protein (CRP or CAP; Gaston *et al.* 1989; Busby and Ebright 1997) homologue, that is a transcription factor (Chin *et al*. 2010). While most Crp proteins bind

cAMP, Clp binds c-di-GMP (Figure 1.5) modulating the expression of a number of genes that are involved in *Xanthomonas* virulence (Chin *et al*. 2010). Another determinant able to sense c-di-GMP is the RxxD domain found by Lee and colleagues in the PelD protein of *P. aeruginosa* (Figure 1.5; Lee *et al.* 2007). PelD is a transmembrane protein encoded by the *pelD* gene belonging to operon required for pellicle production and PEL exopolysaccharide synthesis (see section 1.2.1; Lee *et al.* 2007). Although the exact function of PelD is not known, it is likely to be part of the machinery that synthesizes the PEL exopolysaccharide. PelD has an RxxD motif which is also found in the I sites (inhibition sites) of some DGCs such as PleD (Chan *et al.* 2004). Indeed substitutions to alanine of the arginine and glutamate residues in the RxxD sequence abolishes c-di-GMP binding to PelD. Mutants unable to bind c-di-GMP are also unable to support pellicle formation reiterating the importance of c-di-GMP binding to PelD is for PEL synthesis (Lee *et al.* 2007; Karatan and Watnick 2009). Finally, in addition to binding protein partners, c-di-GMP has also been shown to specifically bind specialized RNA domains (called riboswitches) in order to regulate translation of target mRNAs (Figure 1.5; Sudarsan *et al*. 2008).

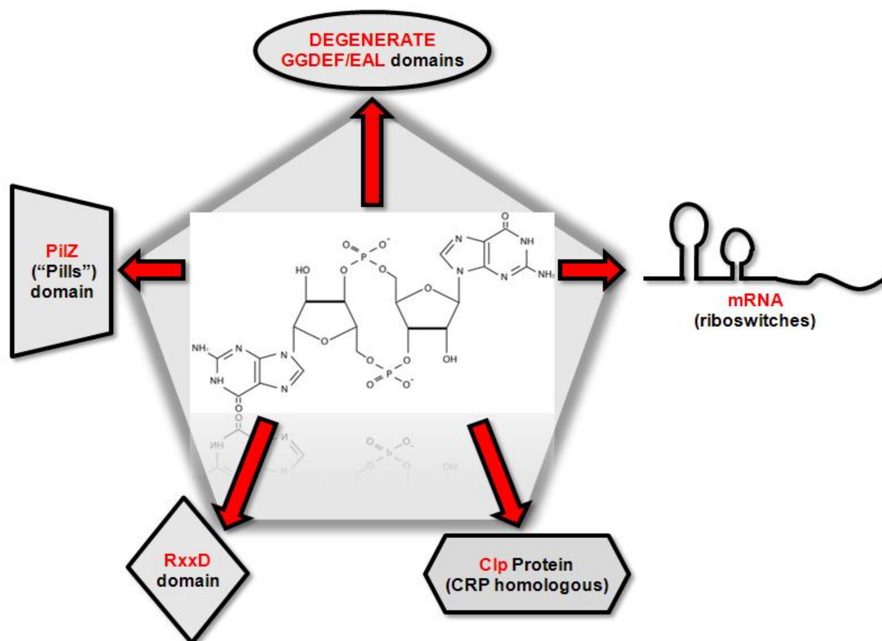


Figure 1.5 c-di-GMP interactions. In literature are described five effectors that are able to bind c-di-GMP: RxxD domain, PilZ ("Pills") domain, degenerate GGDEF/EAL domains, mRNA riboswitches and protein which previously had some other function, such as binding other small nucleic acids (*e.g.* Clp from *X. campestris*).

Thus, the high degree of heterogeneity in c-di-GMP-binding factors (Figure 1.5) allows c-di-GMP to control cellular behavior at different levels:

1. at a **transcriptional level** through binding to transcription factors like *X. campestris* Clp and *P. aeruginosa* FleQ (Hickman and Harwood 2008). In particular FleQ is known to activate expression of flagella biosynthesis genes and also represses transcription of genes including the *pel* operon involved in EPS biosynthesis. In presence of high c-di-GMP intracellular levels FleQ is unable to repress pel operon transcription (Hickman and Harwood 2008)
2. at a **translational level** through interactions with mRNA riboswiches (Sudarsan *et al.* 2008) or mRNA-processing enzymes like polynucleotide phosphorylase (PNPase; Tuckerman *et al*. 2011),
3. at a **post-translational level** by allosteric regulation of enzymatic complexes or other effector molecules such as BcsA (Zogaj *et al.* 2001) and by control of the protein stability (*e.g.* Boehm *et al.* 2009 described a c-di-GMP-dependent degradation of PgaD protein which is involved in poly-*N*-acetylglucosamine biosynthesis in *E.coli*)

GGDEF and EAL proteins in *E. coli*

As shown in Figure 1.7 page 28, *E. coli* K-12 has 29 genes involved in c-di-GMP turnover, which encode, respectively, 12 proteins with GGDEF domains, 11 proteins with EAL domains and 6 proteins that feature both domains (Méndez-Ortiz *et al.* 2006; Sommerfeldt *et al.* 2009; Bohem *et al.* 2009). Given these numbers, it is conceivable that target components and processes controlled by these proteins can be various and different. However, neither c-di-GMP impact on cell physiology nor its effector mechanisms are yet clearly understood. As already mentioned, c-di-GMP can promote biofilm formation. In laboratory strain of *E. coli* c-di-GMP plays a pivotal role in the regulation of curli fibers. Indeed, at least two separate DGC–PDE systems (YdaM–YciR and YegE–YhjH) control the transcription of the *csgDEFG* operon, which encodes for both proteins required for curli assembly and export and for the transcriptional regulator CsgD, which, in turn activates the transcription of the *csgBAC* operon (Figure 1.6; Brombacher *et al.* 2003). Transcription of *csgBAC* operon is also directly stimulates by the DGC YeaP (Sommerfeldt *et al.* 2009). The DGC AdrA (also referred as YaiC), which is expressed under CsgD control during entry into stationary phase (Weber *et al.* 2006; Kader *et al.* 2006; Brombacher *et al.* 2006), is required for cellulose production (Zogaj *et al.* 2001); its function is counteracted by the EAL domain protein YoaD

(Figure 1.6; Brombacher *et al.* 2006). Finally the production of EPS poly-*N*-acetylglucosamine (PNAG, homologous of PIA, described in section 1.2.1) encoded by *pgaABCD* operon is under the control of a DGC-PDE system. Indeed deletion of the DGC coding gene *ydeH,* as well as overproduction of the PDE YjcC reduce PgaD protein stability (Figure 1.6; Bohem *et al.* 2009) and consequently PNAG production.

The role of GGDEF and EAL proteins and their modulation in *E. coli* biofilm formation in response to environmental signals is one of the central aims of my work; it will be described in Chapter III.

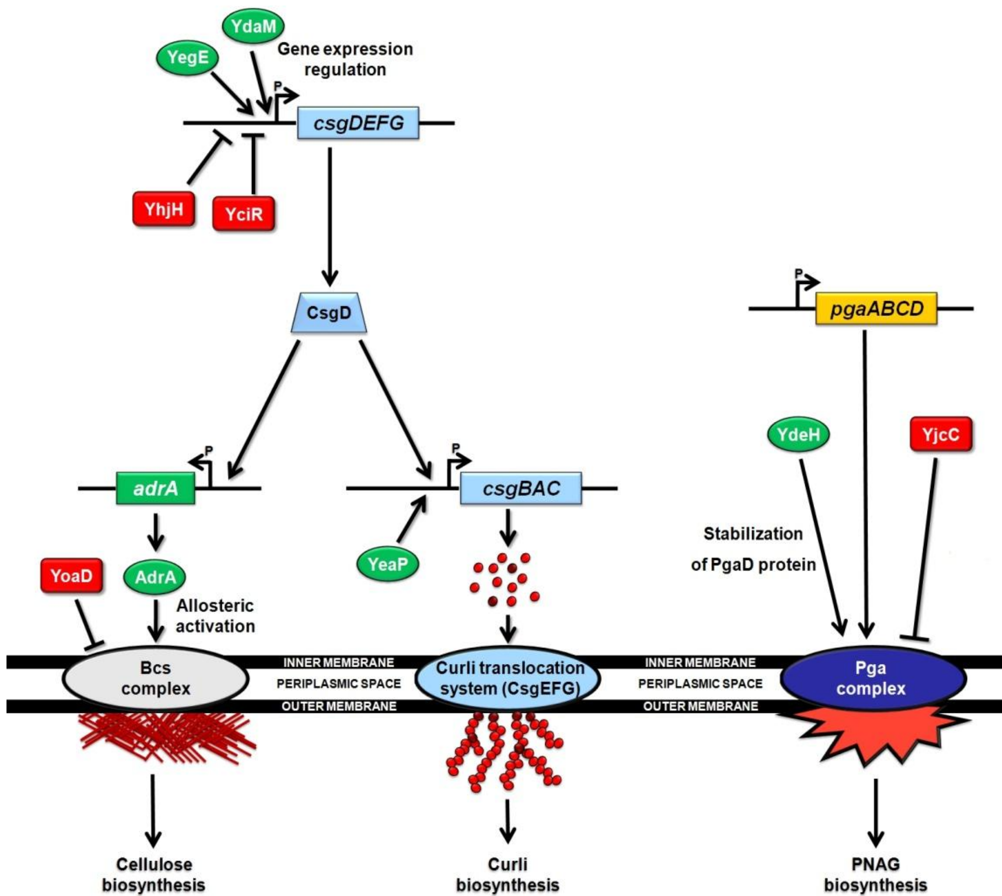


Figure 1.6 Model summarizing DGC- and PDE-mediated production of curli, cellulose and PNAG. Green ellipses indicated DGCs proteins, PDEs are shown as red rectangles.

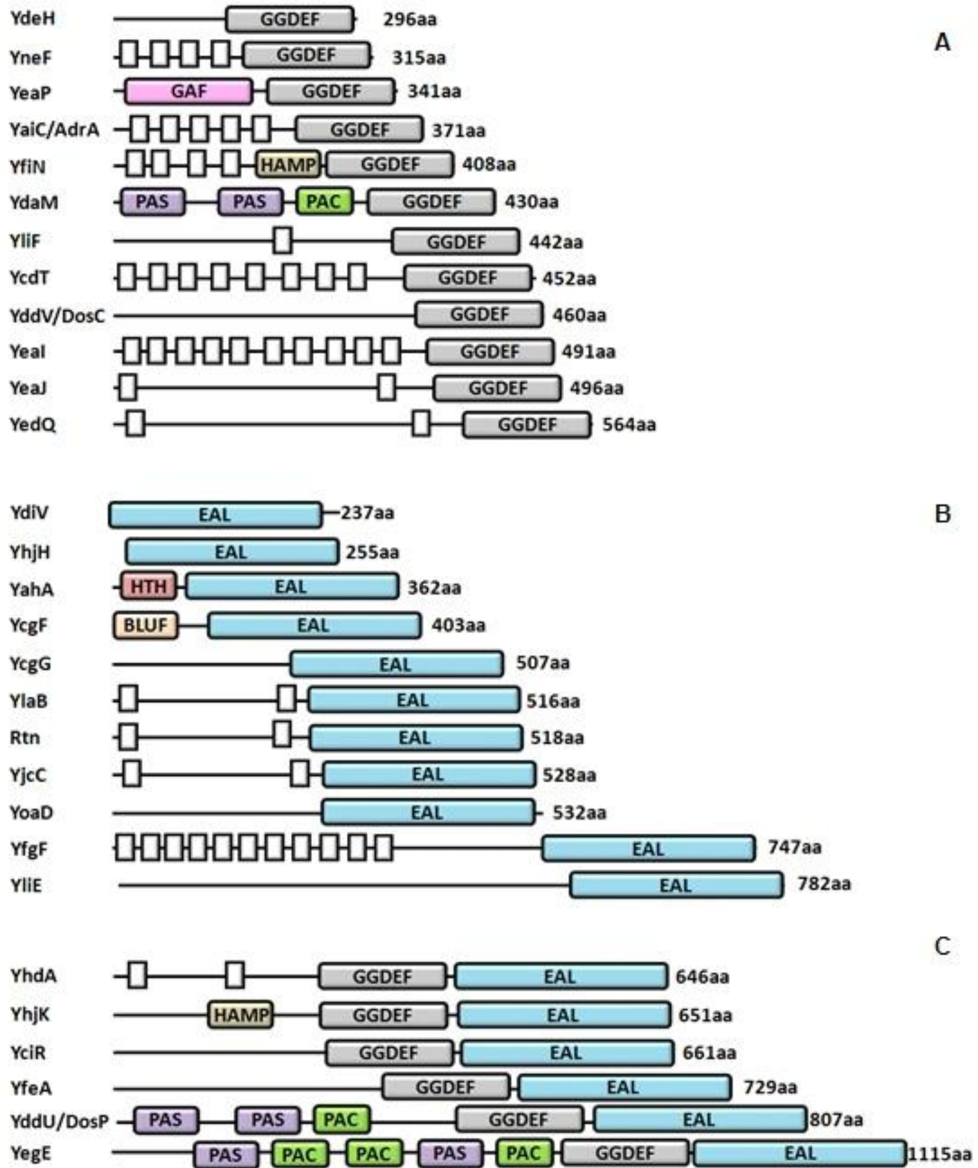


Figure 1.7 Domains organization of the 29 GGDEF/EAL proteins in *E. coli*. **A** Proteins that contain only GGDEF domain (12), **B** Proteins that contain only the EAL domain (11) **C** Proteins that contain both GGDEF and EAL domain (6). White small rectangles represent transmembrane domains. PAS PAC GAF BLUF and HAMP domains are described at page 23; HTH is DNA binding domain. The figure is not in scale

1.4 BIOFILM INHIBITION AND DISPERSAL

Parts of this section's contents have been published in the following article :

Landini P., Antoniani D., Burgess J.G. and Nijland R. (2010). Molecular mechanisms of compounds affecting bacterial biofilm formation and dispersal. *Applied Microbiology and Biotechnology* 86:813-823 (Mini-review).
(http://dx.doi.org/10.1007/s00253-010-2468-8)

Several pieces of evidences have demonstrated that the bacteria in biofilms are considerably less susceptible to antibiotics than their planktonic counterparts (Costerton *et al.* 1995; Anderl *et al.* 2000; Ceri *et al.* 2001; Martinez and Rojo 2011). Although the molecular mechanisms leading to tolerance to antibiotics are not yet fully understood, it has been proposed that the extracellular matrix can affect penetration of antibiotics into bacterial cells. In addition, a dormant metabolic state of a fraction of biofilm cells would also contribute to their decreased antibiotic sensitivity (Lewis 2008). Interestingly, exposure to subinhibitory concentrations of antibiotics and disinfectants can induce biofilm formation in different bacteria (Hoffman *et al.* 2005; Anderson and O'Toole 2008; Nucleo *et al.* 2009): for instance the antimicrobial compound triclosan enhances transcription of cellulose synthesis genes in *S. typhimurium* (Tabak *et al.* 2007). In addition to providing tolerance to antibiotic treatment, biofilms also play an important role in virulence of many pathogenic bacteria. For instance, in *P. aeruginosa*, many virulence factors are expressed during biofilm formation (Wagner *et al.* 2004, Wagner *et al.* 2007). These observations, and the fact that bacterial resistance is undermining the efficacy of currently used antibiotics, indicate that there is a strong need for novel approaches to target pathogenic bacteria growing in biofilms. Therefore, the cellular processes of biofilm formation, maintenance, and dispersal are important targets for the discovery of novel chemical inhibitors. These inhibitors may be used either alone or in combination with conventional antimicrobial agents in antinfective therapies.

1.4.1 TARGET BASED SCREENING

A basic strategy for the discovery of biofilm inhibitors is the direct screening of chemical compounds in biofilm formation assays (Junker and Clardy 2007; Richards *et al.* 2008; Rivardo *et al.* 2009; Peach *et al* 2011). However, such a direct approach also selects for non-specific biofilm inhibitors, such as detergents or biosurfactants, which are not

therapeutically useful. Although these classes of molecules can display significant anti-biofilm activity under laboratory conditions, they often show limited activity, or lack of selective toxicity towards bacteria, if used *in vivo*. In recent years, the improvement in our understanding of the cellular processes controlling bacterial biofilms has allowed the development of target-oriented approaches for the discovery of biofilm inhibitors. Development of target-based screening constitutes a rational and effective strategy for the discovery of biofilm inhibitors. Characterization of quorum sensing (QS described in section 1.3.3) as an important regulatory mechanism in biofilm formation, and thus, as a potential target for antimicrobials (Smith and Iglewski 2003; Njoroge and Sperandio 2009), has led to the development of screening strategies for QS inhibitors. More recently, the search for novel biofilm inhibitors has shown a strong indication that antimicrobial agents affecting nucleotide biosynthesis can be endowed with strong anti-biofilm activity (Attila *et al.* 2009; Ueda *et al.* 2009).

Activity-based screening for QS inhibitors

As described in section 1.3.3, the different chemical nature of signal molecules and of the molecular mechanisms involved in QS would suggest that QS inhibitors can only be directed against either Gram positive or Gram negative bacteria. Search for natural products able to inhibit QS and in particular acylhomoserine lactones (AHLs) biosynthesis has led to the identification of halogenated furanones, produced by the marine alga *Delisea pulchra* (Hentzer *et al.* 2002), and 4-nitro-pyridine-N-oxide (4-NPO) from garlic (*Allium sativum*) cloves (Rasmussen *et al.* 2005). However, furanones show killing activity also against Gram positive bacteria and even Protozoa (Zhu *et al.* 2008; Lönn-Stensrud *et al.* 2009), suggesting that they might target cellular processes other than QS. Indeed, exposure of the Gram positive bacterium *Bacillus subtilis* to furanones triggers induction of stress response genes in a QS-independent manner (Ren *et al.* 2004). Furanones have been identified using activity-based screening in which expression of reporter genes under the control of QS-dependent promoters was measured (Hentzer *et al.* 2002; Rasmussen *et al.* 2005). Further investigation of their mechanism of action showed that furanones bind LasR (one of the regulatory proteins responding to AHLs in *P. aeruginosa*) and act as competitive inhibitors of AHL binding (Hentzer *et al.* 2002). Binding of furanones results in faster degradation of LasR, probably due to destabilization of its conformation (Manefield *et al.* 2002), thus leading to complete inhibition of QS-dependent gene regulation (Hentzer *et al.* 2003). Both furanones and 4-NPO inhibit biofilm formation while not affecting cell growth,

reduce *P. aeruginosa* virulence in experimental infection models, and increase its sensitivity to antibiotics (Hentzer *et al.* 2003). An interesting case of molecules combining antibiotic and anti-biofilm activities are macrolide antibiotics, in particular azithromycin. This antibiotic shows very poor antimicrobial activity against *P. aeruginosa* and other Gram negative bacteria, in particular clinical isolates (Hoffmann *et al.* 2007). However, azithromycin interferes with *P. aeruginosa* biofilm formation (Mizukane *et al.* 1994; Ichimiya *et al.* 1996) by blocking AHL-mediated QS (Tateda *et al.* 2001; Nalca *et al.* 2006). Treatment with azithromycin can attenuate chronic *P. aeruginosa* lung infection and significantly reduce bacterial load in the lungs of Cftr$^{-/-}$ mice, an animal infection model mimicking chronic pneumonia in cystic fibrosis patients (Hoffmann *et al.* 2007). The molecular mechanism of QS inhibition by macrolides has not yet been identified, but it seems likely that they might only affect QS in an indirect fashion through interaction with their primary target, *i.e.*, the ribosome.

Structure-based screening for QS inhibitors

In addition to activity-based assays, an alternative strategy for target-oriented discovery of QS inhibitors is represented by structure-based screening of chemical compounds. This strategy relies on the availability of a growing number of three-dimensional protein structures either predicted by computational biology methods or characterized through biochemical structural analysis. Using molecular modeling programs, it is possible to select potential inhibitors targeting catalytic domains or key amino acid residues for protein activity using virtual screening of small molecules with known structures and chemical properties (Li *et al.* 2008; Kiran *et al.* 2008; Zeng *et al.* 2008; Yang *et al.* 2009). This structure-based approach constitutes a primary virtual screening followed by a secondary activity-based assay using reporter genes controlled by QS-dependent promoters.

Another important application of structure-based screening is provided by drug design, which is not simply the virtual screening of pre-existing molecules, but the tailoring of new, “custom made”, inhibitors based on the structure of a target protein. Proteins involved in QS of Gram negative bacteria, in particular the LasR transcriptional regulator of *P. aeruginosa*, have been used as a target in structure-based screening for biofilm inhibitors. This approach has led to the identification of several compounds showing significant inhibition of QS in *P. areuginosa* (Smith *et al.* 2003; Müh *et al.* 2006; Geske *et al.* 2007; Amara *et al.* 2009).

In Gram positive bacteria, QS directly regulates biofilm maintenance and dispersal, rather than being a factor in its initial formation (Pratten *et al.* 2001; Yarwood *et al.* 2004).

In addition, QS systems of pathogenic Gram positive bacteria, such as the *agr* regulatory system of *S. aureus*, play a fundamental role in the regulation of virulence factors which contributes to the pathogenicity of biofilm-induced infections and are therefore considered targets of great interest for antimicrobials able to interfere with bacterial virulence (Recsei *et al.* 1986; Janzon and Arvidson 1990; Abdelnour *et al.* 1993; Abraham 2006). An interesting mechanism which interferes with biofilm formation in *S. aureus* involves the heptapeptide RIP. This peptide inhibits biofilm formation of *S. aureus in vivo* (Giacometti *et al.* 2003), possibly by blocking the *agr* dependent QS system (Balaban *et al.* 2004). However, the *agr* system might not be RIP primary target since it has also been reported that inhibition of the *agr* system increases biofilm formation (Vuong *et al.* 2003). Although the underlying biology remains unclear, RIP appears to have an effect on biofilm formation, and as such, its structure is an interesting subject for modeling studies aimed at the identification of other biofilm inhibitors. Through structure-based virtual screening using RIP as a template, Kiran *et al*. (2008) identified hamamelitannin, a tannic acid derivative from the bark of *Hamamelis virginiana* (witch hazel). Interestingly, bark extracts of *H. virginiana* are used in natural medicine as astringent and possess weak antibacterial activity (Iauk *et al.* 2003). Hamamelitannin displayed strong inhibition of QS in *S. aureus* and other Gram positive bacteria. Similar to inhibitors of QS in Gram negative bacteria, treatment with hamamelitannin does not result in any detectable growth inhibition of *S. aureus*, but it effectively counteracts *S. aureus* infection in animal models (Kiran *et al.* 2008).

1.4.2 INHIBITORS OF NUCLEOTIDE BIOSYNTHESIS AND DNA REPLICATION AS ANTI-BIOFILM AGENTS

It has recently been reported that fluorouracil, which blocks DNA replication through inhibition of nucleotide biosynthesis, can prevent biofilm formation at concentrations not affecting planktonic cell growth (Attila *et al.* 2009; Ueda *et al.* 2009). This observation indicates that nucleotide biosynthesis inhibitors might be particularly effective against biofilms and suggests that a decrease in cellular nucleotide pools negatively affects biofilm formation. This has been confirmed by reports showing that mutations in nucleotide biosynthesis gene negatively affect biofilm formation (Haugo and Watnick 2002; Ueda *et al.* 2009, Garavaglia *et al.* 2012). Consistent with this finding, surface adhesion is impaired by mutations in genes responsible for nucleotide biosynthesis (Ueda *et al.* 2009). Inhibition of nucleotide biosynthesis might block the production of modified nucleotides which act as signal molecules for biofilm formation and stimulate their degradation and recycling in

nucleotide triphosphate biosynthesis for DNA and RNA. Another possibility might be that an even partial inhibition of nucleotide biosynthesis, such as observed at fluorouracil concentrations not affecting bacterial growth, might result in shortage of deoxyribonucleotides for DNA replication. The bacterial cell may then react by abolishing “non-essential” DNA synthesis, such as production of extracellular DNA which is essential for biofilm formation in some bacterial species (Whitchurch *et al.* 2002).

1.4.3 REMOVAL OF BACTERIAL BIOFILMS BY PROMOTING THEIR DISPERSAL

Biofilm dispersal is a naturally occurring process which may represent a mechanism to escape starvation or other negative environmental conditions within a biofilm, giving bacterial cells the opportunity to migrate to a more favorable environment. In order to promote their dispersal, biofilm cells need to produce enzymes able to degrade the EPS matrix that surrounds them. To do this, a wide variety of EPS-degrading enzymes are used. *P. aeruginosa* secretes alginate lyase (Boyd and Chakrabarty 1994), whereas the oral pathogen *Aggregatibacter actinomycetemcomitans* (Kaplan *et al.* 2003) produces Dispersin B, a protein that specifically hydrolyzes the glycosidic linkages of poly-β-1,6-*N*-acetylglucosamine (PNAG, homologous of PIA, section 1.2.1), an EPS that functions as an important biofilm determinant in both Gram negative and Gram positive microorganisms (Cramton *et al.* 1999; Wang *et al.* 2004a).

Biofilm dispersal in *X. campestris* can be triggered by the addition of DSF that, as mentioned before, acts as a diffusible QS signal (Dow *et al.* 2003; Wang *et al.* 2004b). DSF triggers expression of the *manA* gene, encoding endo- β-1,4-mannanase, which results in EPS degradation and biofilm dispersal (Dow *et al.* 2003). It has recently been reported that a monounsaturated fatty acid produced by *P. aeruginosa*, cis-2-decenoic acid, can induce cell detachment from biofilms; interestingly, cis-2-decenoic acid displays biofilm-dispersing effects on both Gram positive and Gram negative bacteria (Davies and Marques 2009). The enzyme lysine oxidase has recently been implicated in the dispersal of biofilms in a number of Gram negative bacteria. This enzyme has been shown to mediate cell death due to the production of hydrogen peroxide (Mai-Prochnow *et al.* 2008).

Compound	Mechanism of biofilm inhibition	Other known biological effects	Identification	References
Furanones and structural analogues	AHL binding by LasR protein	Antimicrobial activity on Gram positive bacteria	Activity-based screening	Hentzer *et al.* 2002; Müh *et al.* 2006
Azithromycin	Inhibition of LasR-dependent gene expression	Protein synthesis inhibitor	Evaluation of antimicrobial activity	Nalca *et al.* 2006 Hoffmann *et al.* 2007
4-NPO	Inhibition of LasR-dependent gene expression	None	Activity-based screening	Rasmussen *et al.* 2005
Hamamelitannin	RIP analogue (RNAIII inhibitor)	None	Structure-based virtual screening	Kiran *et al.* 2008
Fluorouracil	Inhibition of AriR biofilm regulatory protein	Inhibition of nucleotide biosynthesis	Activity-based screening	Attila *et al.* 2009
Dispersin B	Enzymatic degradation of biofilm matrix	None	Genetic screening for mutants in biofilm formation	Kaplan *et al.* 2003

Table 1.2. A selection of biofilm inhibitors found in activity- and structure- based screening.

1.4.4. NEW STRATEGIES FOR BIOFILM INHIBITON AND DISPERSAL

In October 2009, Cegelsky *et al.* published that ring-fused 2-pyridones inhibited curli (one of the major adhesion factor in *E.coli*) biogenesis. In particular Ring-fused 2-pyridones prevented the *in vitro* polymerization of the major curli subunit protein CsgA. (Cegelsky *et al.* 2009). More recently, in April 2010, Kolodkin-Gal and colleagues showed that in *Bacillus subtilis* a mixture of D-leucine, D-methionine, D-tyrosine, and D-tryptophan causes the release of amyloid fibers that linked cells in the biofilm together (Kolodkin-Gal *et al.* 2010). The mixture of the 4 D-amino acid could act at nanomolar concentrations. Mutants able to form biofilms in the presence of D-amino acids contained alterations in a protein (YqxM) required for the formation and anchoring of the fibers to the cell. In addition D-amino acids also prevented biofilm formation by *S. aureus* and *P. aeruginosa*. Since D-acids are produced by many bacteria they may be a widespread signal for biofilm disassembly (Kolodkin-Gal *et al.* 2010)

However, despite promising results, the quest for anti-biofilm agents which either alone or in combination with conventional antimicrobials, could results effective in the treatment of biofilm-related infections, is still open. In the first part of my thesis, I set up a series of microbiological assays for the efficient screening of molecules inhibiting c-di-GMP biosynthesis.

CHAPTER II

MONITORING OF CYCLIC-DI-GMP BIOSYNTHESIS VIA-ASSAYS SUITABLE FOR HIGH-THROUGHPUT SCREENING OF BIOFILM INHIBITORS

Some of the results described in this chapter have been published in the following publication:

Antoniani D., Bocci P., Maciag A., Raffaelli N. and Landini P. (2010). Monitoring of diguanylate cyclase activity and of cyclic-di-GMP biosynthesis by whole-cell assays suitable for high-throughput screening of biofilm inhibitors. *Appl. Microbiol. Biotechnol.* 85:1095-1104.
(http://dx.doi.org/10.1007/s00253-009-2199-x)

2.1 INTRODUCTION

Transition from planktonic cells to biofilm is regulated by environmental and physiological signals, relayed to the bacterial cell by signal molecules or "second messengers". A second messenger, bis-(3′,5′)-cyclic diguanylic acid, better known as cyclic-di-GMP (c-di-GMP), plays a pivotal role in several processes linked to biofilm formation and maintenance, such as production of EPS and adhesion factors (Tal *et al.* 1998; Kader *et al.* 2006; Weber *et al.* 2006). c-di-GMP-related genes are widely conserved among bacteria but are only sporadically present in Eukarya and totally absent in animal species (Galperin 2004), which makes enzymes involved in c-di-GMP biosynthesis interesting as targets for antimicrobial or anti-biofilm agents. The discovery of inhibitors of novel DGCs (the class of proteins which synthesize c-di-GMP) can be greatly facilitated by the development of assays suitable for high-throughput screening (HTS) of chemical compounds. HTS must be based on simple and reliable assays, suitable for automation and, whenever possible, performed in living cells, in order to select compounds able to cross the membrane barrier and to show activity *in vivo*. However, to our knowledge, no rapid methods for screening for DGC inhibitors based on their mode of action have yet been described in literature. In this chapter, I describe the exploitation of a suite of well-established microbiological assays as a screening approach for inhibitors of DGC enzymes. As a primary screening method, we employed a Congo red (CR) assay, which provides a simple, qualitative, whole-cell assay to test DGC activity in living cells. Rapid secondary screening methods are provided by the crystal violet assay for semi-quantitative measurement of biofilm formation and by a quantitative reporter gene assay measuring expression of curli-encoding genes as a function of DGC activity. Screening of a chemical library using this strategy led to the identification of two inhibitors of c-di-GMP biosynthesis: sulfathiazole and azathioprine.

2.2 RESULTS

2.2.1 RATIONAL DESIGN OF A CONGO RED-BASED MICROBIOLOGICAL ASSAY FOR DIGUANYLATE CYCLASES (DGCs) INHIBITORS

CR is a diazo-dye with strong affinity for amyloid fibers (Bennhold 1922), such as curli fibers produced by Enterobacteria (Olsén *et al.* 1989). In addition, CR can also bind polysaccharides (EPS) such as cellulose (Zogaj *et al.* 2001; Da Re and Ghigo 2006). It is well established that, in Enterobacteria, production of both curli and cellulose depends on c-di-GMP biosynthesis and is mediated by specific DGCs: YdaM is required for curli production in *E. coli* (Weber *et al.* 2006; Sommerfeldt *et al.* 2009), while AdrA is necessary for cellulose biosynthesis in *Salmonella* (Zogaj *et al.* 2001). In *E. coli* laboratory strains such as MG1655, red colony phenotype on CR-supplemented medium is totally dependent on curli production, due to low levels of cellulose production, and mutants in curli-encoding genes display a white phenotype on CR medium (Gualdi *et al.* 2008; see also Figure 2.2A page 44). Thus, since in *E. coli* the red phenotype on CR medium depends on curli production, which in turn requires c-di-GMP biosynthesis by YdaM (Weber *et al.* 2006), exposure of *E. coli* to DGC inhibitors would result in a white phenotype on CR medium, providing an easy screening assay. However, complex regulation of curli expression, which, albeit strongly dependent on c-di-GMP, is also under the control of various regulators and several physiological signals (see Chapter I, sections 1.3.1, 1.3.4, 1.3.5), makes a screening based on curli production unsuitable for search of DGC inhibitors. Thus, we developed a strain that could act as a suitable reporter in a screening assay for DGC inhibition. To this, the AM70 a *csgA* mutant derivative of *E. coli* MG1655 (Table 2.3) unable to produce curli and showing white phenotype on CR medium (see Figure 2.2A page 44), was transformed with a multicopy plasmid carrying the DGC-encoding *adrA* gene (pTOPOAdrA$_{wt}$). The resulting strain shows curli-independent red phenotype on CR medium as a direct result of DGC activity (see Figure 2.2A page 44). Indeed, from literature data (Zogaj *et al.* 2001), we expected *adrA* overexpression to activate cellulose production, thus resulting in a red phenotype on CR medium even in a *csgA* mutant of *E. coli* unable to produce curli. A possible additional advantage of using an AdrA-overexpressing strain is that the *adrA* gene can be placed under the control of an inducible promoter (*e.g.*, the isopropyl β-*D*-1-thiogalactopyranoside (IPTG)-inducible P*lac* promoter) and expressed at different

levels either in the presence or in the absence of the inducer molecule. A first round of screening would then be performed on strains expressing the target protein at low concentrations (no IPTG induction), and chemical compounds showing inhibition of red coloring on CR medium can be tested on strains induced with IPTG (full AdrA overexpression). Increased amounts of the target protein should result in the need of higher inhibitor concentrations to prevent red coloring on CR plates, thus providing further limitation in selection of false positives, as represented in Figure 2.1. However in the pTOPO plasmid, full expression from the P*lac* promoter seems to take place even in absence of IPTG induction (data not shown), and the experiments described in the next section were therefore only performed in the absence of IPTG.

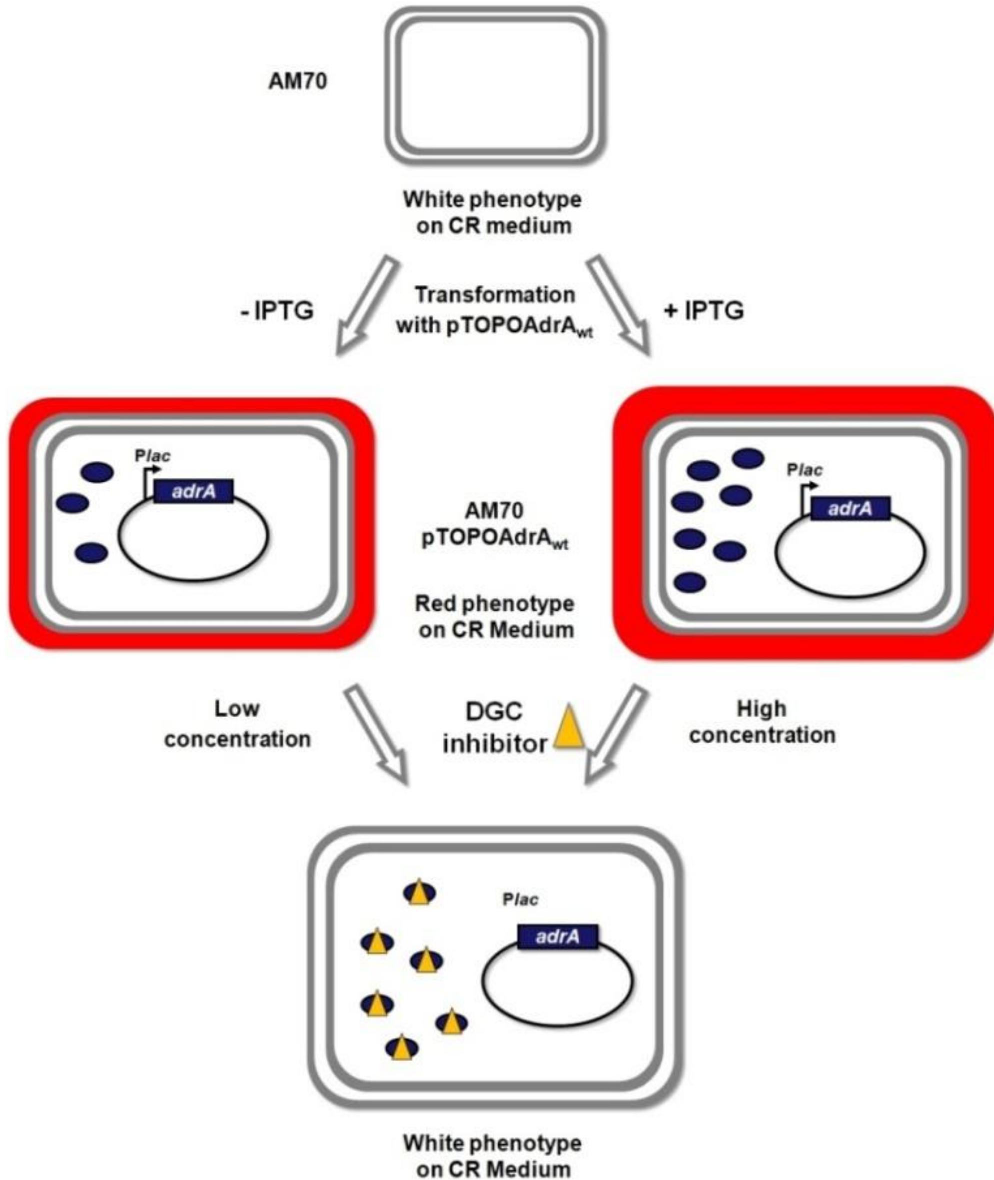


Figure 2.1 Design of the *Escherichia coli* reporter strain used in the Congo red (CR) assays. The Δ*csgA* AM70 strain is transformed with the pTOPOAdrA$_{wt}$ plasmid; growth either in the presence or in the absence of isopropyl β-D-1-thiogalactopyranoside can lead to different AdrA expression levels with consequent production of cellulose binding CR (indicated by the additional extracellular layer different thickness levels only intend to indicate isopropyl β-D-1- thiogalactopyranoside-induced and uninduced cells and are not representative of actual cellulose amounts produced in either condition). Presence of a diguanylate cyclase inhibitor would block AdrA activity resulting in a white phenotype on CR medium.

2.2.2 VALIDATION OF AM70/pTOPOAdrA$_{wt}$ AS AN INDICATOR STRAIN FOR SCREENING OF DGC INHIBITORS

In order to use AM70 strain transformed with the pTOPOAdrA$_{wt}$ plasmid (AM70/pTOPOAdrA$_{wt}$) as the indicator strain in the screening for DGC inhibitors, we needed to verify whether it can display a red phenotype on CR medium and form biofilm in a manner dependent on AdrA DGC activity. Expression of the *adrA* gene from the pTOPOAdrA$_{wt}$ plasmid in the absence of IPTG induction confers a red phenotype on CR medium to AM70, while transformation of the same strain with the pTOPO control vector does not affect its white phenotype (Figure 2.2A). Since AdrA expression stimulates cellulose production and CR binding in *Salmonella* (Zogaj *et al.* 2001); AdrA-dependent red phenotype in a curli-deficient strain should depend on cellulose production. Indeed, pTOPOAdrA$_{wt}$ fails to confer a red phenotype on CR medium to a *csgA/bcsA* double mutant strain unable to produce either curli or cellulose (Figure 2.2A). IPTG induction did not result in any detectable change in AM70/pTOPO or AM70/pTOPOAdrA$_{wt}$ phenotype on CR medium (data not shown). As a further verification that *adrA* overexpression results in cellulose production, we plated the AM70 strain transformed either with pTOPOAdrA$_{wt}$ or pTOPOAdrA$_{GGAAF}$ or with the pTOPO control vector on Calcofluor-supplemented plates. Calcofluor (CF) is a fluorescent whitener which binds specifically to β-glucans such as cellulose and chitin; CF binding to cellulose can be visualized by exposure to UV light (Perry and Miller 1989). As expected, only the AM70/pTOPOAdrAwt strain showed fluorescence upon UV light exposure, indicating that AdrA overexpression does indeed result in activation of cellulose production (Figure 2.2B). To confirm that the adrA-induced phenotypic changes in CR- and CF-supplemented media were indeed due to AdrA DGC activity, we transformed AM70 with a plasmid carrying a mutated allele of the *adrA* gene, encoding an AdrA protein in which the GGDEF amino acid sequence of the DGC catalytic site was changed to GGAAF (pTOPOAdrA$_{GGAAF}$). Substitution to alanine of any residue in the GGDEF motif strongly affects DGC activity thus impairing c-di-GMP biosynthesis (Simm *et al.* 2004; Malone *et al.* 2007; De *et al.* 2008; Jonas *et al.* 2008). The intracellular c-di-GMP concentration both in MG1655 cells and in the cells transformed with either pTOPO or pTOPOAdrA$_{wt}$ or pTOPOAdrA$_{GGAAF}$ was determined by HPLC analysis. As shown in Figure 2.2C, c-di-GMP was clearly detected in cells expressing AdrA protein at a concentration of 360 nmol/g (dry weight), in agreement with the levels measured in an AdrA-overexpressing strain of *Salmonella* (Simm *et al.* 2004). Intracellular c-di-GMP

concentrations in MG1655 and in MG1655 carrying the control vector contained 3.5 and 2.4 nmol/g c-di-GMP, respectively.

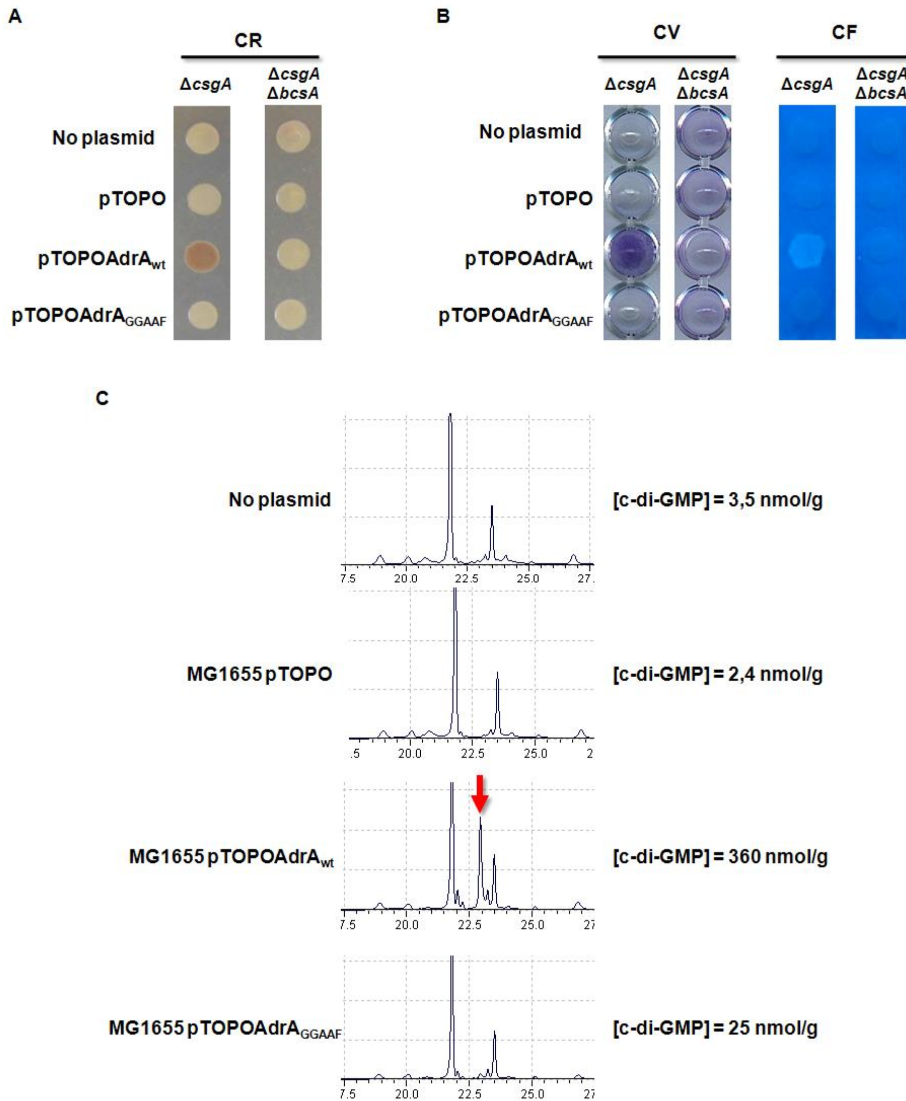


Figure 2.2 A Effects of the expression of AdrA (either *wild type* or GGAAF mutant) in AM70 (MG1655 Δ*csgA*, curli-deficient) and AM73 (MG1655 Δ*csgA* Δ*bcsABZC*, curli- and cellulose-deficient) on Congo red binding (CR). **B** Effects of expression of AdrA$_{wt}$ and AdrA$_{GGAAF}$ proteins on biofilm formation (measured with the crystal violet assay) and on Calcofluor (CF) binding. Semi-quantitative evaluation of biofilm in crystal violet (CV) assays gave adhesion values of 19.6 for AM70/ pTOPOAdrA$_{wt}$ and of 0.53 for AM70/pTOPO. **C** Determination of c-di-GMP biosynthesis in AdrA$_{wt}$ and AdrA$_{GGAAF}$ protein-expressing strains by high-performance liquid chromatography. The peak corresponding to c-di-GMP is marked by a red arrow; the peak with a retention time of 21.8 min corresponds to NAD, while the peak at 23.5 min was not identified.

Expression of the $AdrA_{GGAAF}$ protein resulted in an intracellular c-di-GMP concentration of 25 nmol/g, *i.e.*, a >90% reduction of intracellular c-di-GMP compared to MG1655 transformed with pTOPOAdrAwt (Figure 2.2C). Expression of the $AdrA_{GGAAF}$ protein failed to induce cellulose production when expressed in AM70 (Figure 2.2A, and 2.2B), consistent with its poor DGC activity.

In order to test if AdrA-mediated cellulose production would result in biofilm formation, we performed surface adhesion experiments using the crystal violet (CV) assay (Dorel *et al.* 1999), which clearly showed increased adhesion to polystyrene microtiter plates by AM70 transformed with $pTOPOAdrA_{wt}$ but not with $pTOPOAdrA_{GGAAF}$ (Figure 2.2B).In addition AdrA overexpression didn't stimulate biofilm formation in the double mutant Δ*csgA*/Δ*bcsA* (Figure 2.2B) Thus, AM70/$pTOPOAdrA_{wt}$ phenotypes on CR- and CF-supplemented plates, as well as its ability to form biofilm, totally depend on AdrA DGC activity. Our results clearly suggest that the AM70/$pTOPOAdrA_{wt}$ strain can be a suitable reporter strain to measure inhibition of DGC activity via determination of its phenotype on CR medium. One possible drawback of the CR screening relies on the fact that assays on solid medium are usually not amenable for HTS, mainly due to the amount of chemical compounds required for standard plates and to difficulties in automation of the assay. To overcome these limitations, we miniaturized the CR assay in 96-well microtiter plates: 200 µl of CR medium prior to solidification are distributed using a multi-channel pipette in each well; after solidification, 5 µl of an overnight culture of AM70/$pTOPOAdrA_{wt}$ are layered on top of the solidified CR medium. The chemicals to be tested can be added in solution (5–10 µl) at various concentrations to the bottom of the microtiter plate wells prior to the addition of CR medium. This "miniaturized CR assay" was used to screen a chemical library for DGC inhibitors.

2.2.3 DGC-DEPENDENT GENE EXPRESSION ASSAYS

The assays described in the previous sections can be used to select for inhibitors of biofilm formation dependent on DGC activity. However, chemical compounds able to affect CR phenotype and surface adhesion in crystal violet assays might target steps in biofilm formation other than c-di-GMP biosynthesis, or they might be capable of non-specific binding to the cell surface with consequent alteration of its physico-chemical properties. Thus, a strategy for selection of DGC inhibitors should include a tertiary screening assay that

can assess inhibition of DGC activity inside the bacterial cell directly. In addition to acting as an allosteric activator of the cellulose biosynthetic proteins, the AdrA protein can activate transcription of curli-encoding *csg* genes when overexpressed in *S. enterica* (Kader *et al.* 2006). Another DGC protein, YdaM, controls expression of curli-encoding *csg* genes through its DGC activity in *E. coli* (Weber *et al.* 2006; Pesavento *et al.* 2008). We transformed an MG1655 derivative carrying a *csgA::uidA* chromosomal fusion (PHL856, Gualdi *et al.* 2008), either with pTOPOAdrA$_{wt}$ or with pTOPOYdaM. *uidA* is a reporter gene encoding β-glucuronidase, whose enzymatic activity can easily be monitored with a colorimetric assay (Bardonnet and Blanco 1992); the *csgA::uidA* fusion leads to β-glucuronidase production in response to transcription of the *csgBAC* operon, encoding curli structural subunits (Prigent-Combaret *et al.* 2001). β-glucuronidase experiments performed on overnight cultures grown in M9 Glu-sup medium at 30°C show that *csgBAC* transcription is activated by both AdrA (ca. 4-fold) and YdaM (ca. 6.5-fold; Figure 2.3). Thus, measurement of DGC-dependent *csgBAC* transcription by β-glucuronidase assays provides a convenient method to test DGC inhibition by compounds showing activity in the CR and biofilm formation assays.

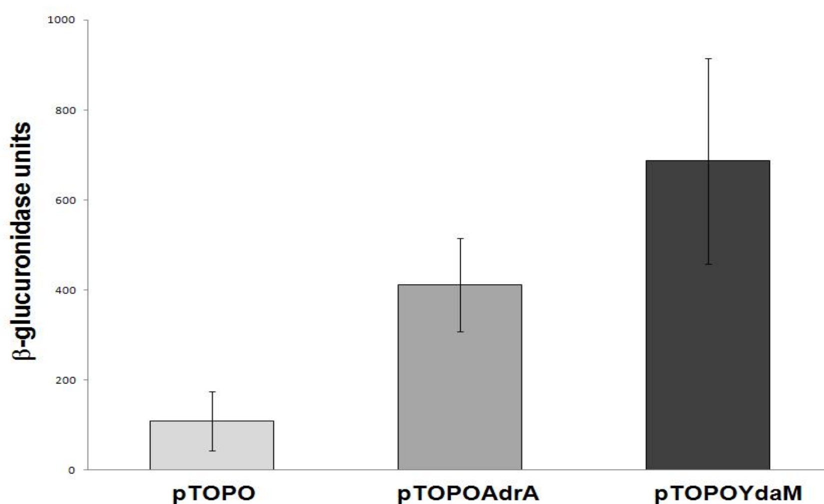


Figure 2.3 β-glucuronidase assay on the PHL856 (MG1655 *csgA::uidA-kan*) strain transformed with pTOPO, pTOPOAdrA$_{wt}$, or pTOPOYdaM plasmids. Average values were 109, 412, and 687 units, respectively. β-glucuronidase activity was determined on overnight cultures grown in M9 Glu-sup at 30°C. Results are the average of four different experiments; error bars are shown.

2.2.4 IDENTIFICATION OF THE ANTIMETABOLITE SULFATHIAZOLE AS INHIBITOR OF c-di-GMP BIOSYNTHESIS

The screening strategy described in the previous sections consists of a qualitative primary assay based on a color phenotype in the miniaturized CR assay, followed by the semi-quantitative crystal violet assay to assess inhibition of biofilm formation, and by the β-glucuronidase reporter assay to verify inhibition of DGC enzymes inside the bacterial cell. Chemical compounds showing inhibitory activity in all three assays can then be tested for their ability to inhibit c-di-GMP biosynthesis in bacterial cells by HPLC. Thus, we screened the Prestwick chemical library (http://www.prestwickchemical.fr/index.php?pa=26), from Prestwick Chemicals. This library contains 1,120 chemical compounds with known biological activities, already tested for bioavailability and safety in humans, based on the Selective Optimization of Side Activities (SOSA) criteria for the identification of novel biological activities by known drugs (Wermuth 2006). One molecule, sulfathiazole, caused strong discoloration of AM70/pTOPOAdrA$_{wt}$ red phenotype when added to the CR medium already at the lowest concentration tested (2 μg/ml, corresponding to 7.8 μM; data not shown) and did not affect bacterial growth up to 50 μg/ml, the highest concentration tested in CR assays. Determination of sulfathiazole MIC in liquid media showed bacterial growth inhibition at ca. 70 μg/ml (275 μM, Table 2.1) sulfathiazole, *i.e.*, at concentrations 35-fold higher than those inhibiting AM70/pTOPOAdrA$_{wt}$ red phenotype on CR medium. Concentrations as low as 5.8 μM inhibited biofilm formation in crystal violet assays (Table 2.1), thus suggesting that the sulfathiazole effect on CR phenotype correlates with its ability to prevent biofilm formation.

Assays	Inhibition by Sulfathiazole (μM)
Biofilm formation (crystal violet assay)[A]	IC_{50}^{*} = 5,8 ± 0,63
csgBAC gene expression (β-glucuronidase assay)[B]	IC_{50}^{*} = 3,9 ± 0,82
c-di-GMP biosynthesis (HPLC)[C]	IC_{50}^{*} = 4,6
Bacterial growth inhibition[D]	275 ± 47

Table 2.1 IC50* =sulfathiazole concentration inhibiting the reaction by 50%
[A]Average of three experiments: control values for AM70/pTOPOAdrAwt = 19.2, for AM70/pTOPO = 0.46 adhesion units [B]Average of three independent experiments: control values for AM70/pTOPOYdaM$_{wt}$ = 673, for AM70/pTOPO = 91 β-glucuronidase units
[C]Average of two determinations with very similar values: control values for AM70/pTOPOYdaM$_{wt}$ = 341, for AM70/pTOPO = 3.1 nmol/g dry weight
[D]Average of three independent experiments. Values determined by visual inspection

Finally, β-glucuronidase reporter assays showed that sulfathiazole was able to inhibit both AdrA- and YdaM-dependent stimulation of *csgA* gene expression by 50% at 3.9 μM (Table 2.1) and by 90% at 7.8 μM (not shown), similar to the concentration needed to prevent cellulose production and biofilm formation. To verify whether the effects of sulfathiazole on CR phenotype, biofilm formation, and curli-encoding gene expression correlated with inhibition of c-di-GMP biosynthesis, we measured intracellular c-di-GMP concentrations both in the absence and in the presence of different sulfathiazole concentrations by HPLC in the MG1655/pTOPOYdaM strain.
As shown in Figure 2.2C and Table 2.1 treatment with sulfathiazole resulted in clear inhibition of c-di-GMP biosynthesis: 50% reduction in c-di-GMP intracellular levels were observed at 4.6 μM sulfathiazole, similar to the concentrations needed to inhibit biofilm formation and activation of *csgA* gene expression (Table 2.1). Complete inhibition of c-di-GMP production was observed at 20 μM sulfathiazole (data not shown).

2.2.5 THE REPORTER STRAIN PHL565W/pTOPOWspR

In addition to expression of *E.coli* genes encoding DGCs such as AdrA and YdaM it is interesting to performed screening assays using *E. coli* strains expressing DGCs from different pathogenic bacteria. Indeed, c-di-GMP is involved in virulence mechanism in various Gram negative pathogens (Cotter and Stibitz 2007; Ryan and Dow 2010) In *P. aeruginosa*, for instance, c-di-GMP-mediated biofilm formation is an important factor in host colonization and appears to play a major role in chronic diseases such as lung infection in cystic fibrosis patients (Häussler 2004). We cloned and overexpressed in *E. coli* the DGC-encoding *wspR* gene from *P. aeruginosa*. In *P. fluorescens*, DGC-activity by WspR is connected with overproduction of acetylated cellulose, biofilm formation and the so called wrinkly spreader (WS) phenotype (Malone *et al.* 2007; Spiers 2007); although it is not clear whether WspR induces cellulose production in *P. aeruginosa,* experimental evidences indicates that WspR plays a key role in a complex network involved in biofilm formation (Güvener and Harwood 2007; Moscoso *et al.* 2011). In the AM70 strain, used in previous experiments, overexpression of WspR only leads to pale pink/orange phenotype on CR medium, and stimulates the formation of a very thin biofilm in microtiter plates (Figure 2.4) Thus we tested different strains for WspR overexpressions. We found that WspR induced stronger phenotypic changes in PHL565W an *E. coli* MG1655 derivative that forms white colonies on CR medium (Figure 2.4).

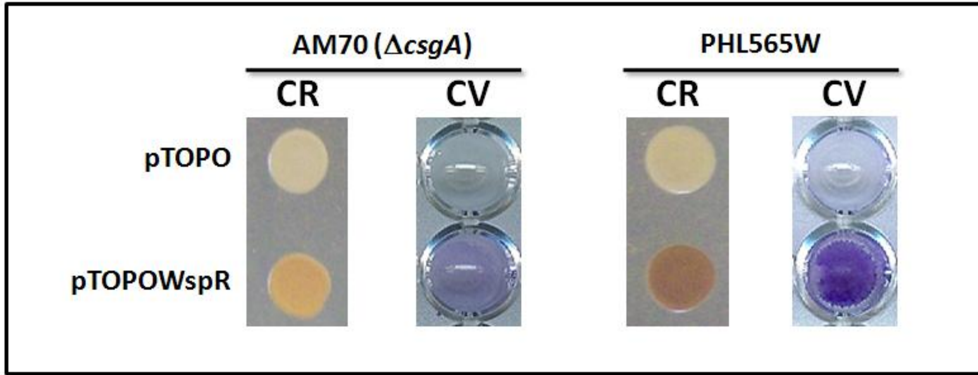


Figure 2.4 Effects of the expression of WspR in AM70 (MG1655 curli deficient strain) and in PHL565W (MG1655 spontaneous mutant unable to express the *rpoS* gene) on Congo red binding (CR) and on biofilm formation measured with the crystal violet assay (CV). Semi-quantitative evaluation of biofilm in crystal violet assays gave adhesion values of 0.57 for AM70/pTOPO, 2,58 AM70/pTOPOWspR, 0.43 for PHL565W/pTOPO and of 22 for PHL565W/pTOPOWspR.

The PHL565W white phenotype on CR medium is due to lack of expression of the *rpoS* gene (W=white on Congo Red; called PHL565 in Gualdi *et al.* 2007), which encodes the σ^S protein, an alternative sigma factor necessary for the expression of curli-encoding genes. Overexpression of WspR in PHL565W can confer a red phenotype to the colony on CR medium (Figure 2.4); since in PHL565W are functional all the genes required for curli biosynthesis, the red phenotype might be depends on WspR-mediated curli overproduction. Consistent whit this possibility WspR is able to stimulate biofilm formation in *E. coli* strain PHL565W as expected by *E. coli* strains proficient in curli production (Figure 2.4).

The intracellular c-di-GMP concentration in PHL565W cells transformed with either pTOPO or pTOPOWspR was determined by HPLC analysis: c-di-GMP was only detected in PHL565W/pTOPOWspR at a concentration of 30 nmol/g (data not shown).

Our results are consistent with a role for WspR as a diguanylate cyclase able to induce cellulose production in several *Pseudomonas* species (Spiers *et al.* 2003; Ude *et al.* 2006). Moreover, De and colleagues demonstrated that overexpression of $WspR_{wt}$ can cause a red colony phenotype in a manner dependent on its DCG activity in *E. coli* BL21 (De *et al.* 2008). Thus, in addition to AM70/pTOPOAdrA, also PHL565W pTOPOWspR can be consider a convenient reporter strain in which red phenotype strictly depends on the DGC activity of WspR.

Finally we decided to test the effect of WspR on *csgBAC* transcription. Using the same rationale described in 2.2.3 we transformed *E. coli* PHL856 (MG1655 carrying a *csgA::uidA*

chromosomal fusion) with pTOPOWspR. As shown in Figure 2.5 WspR overexpression resulted in activation of *csgBAC* transcription (ca. 6-fold Figure 2.5).

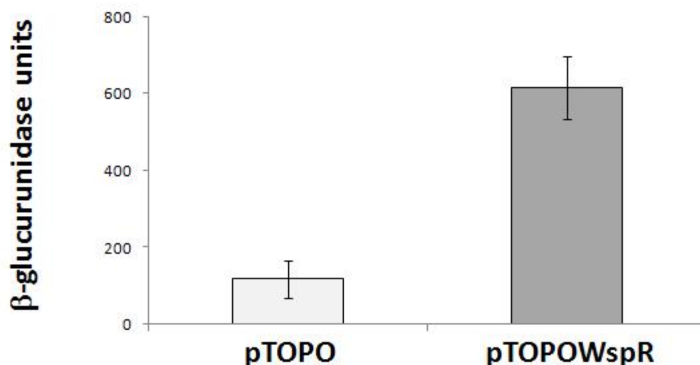


Figure 2.5 β-glucuronidase assay on the PHL856 (MG1655 *csgA::uidA-kan*) strain transformed with pTOPO and pTOPOWspR plasmids. Average values were 107 and 615 units, respectively. β-glucuronidase activity was determined on overnight cultures grown in M9 Glu-sup at 30°C. Results are the average of four different experiments; error bars are shown.

2.2.6 IDENTIFICATION OF ANOTHER INHIBITOR OF c-di-GMP BIOSYNTHESIS: AZATHIOPRINE

Using the reporter strain PHL565W/pTOPOWspR and the methodology described in the previous sections we screened the Prestwick Chemical libraries for possible inhibitors of WspR. We found that, in addition to sulfathiazole, already identified in our previous screening, another compound, namely azathioprine, resulted in decoloration of PHL565W/pTOPOWspR on CR medium at a concentration of 180 μM (50 μg/ml). In addition, azathioprine can prevent PHL565W/pTOPOWspR biofilm formation (IC_{50}=383 μM; Table 2.2). In our conditions, azathioprine was unable to affect bacterial growth, showing an MIC in liquid medium higher than 256 μg/ml corresponding to 923 μM (Table 2.2). As described in the previous section, overexpression of WspR in PHL565W led to a concentration of c-di-GMP of 30nmol/g; exposure to azathioprine clearly reduced the intracellular concentration of c-di-GMP: a reduction by ca. 50% in c-di-GMP intracellular level (Table 2.2) was observed at 180μM, *i.e.*, the same concentration needed to cause the loss of red phenotype in the CR-binding assay.

Azathioprine is an analogue of purine bases (see Figure 2.6) and is thought to act as a pro-drug which, is metabolized into the active 6-mercaptopurine (6-MP, Sandborn 1998). Thus,

we tested 2-amino-6-mercaptopurine hydrate (6-MP-riboside) a derivative of 6-MP in our screening assays. We used 6-MP-riboside since it showed better solubility proprieties than 6-MP. 6-MP-riboside caused loss of red phenotype in PHL565W/pTOPOWspR already at 2μg/ml (corresponding to 6,7μM) and prevented biofilm formation at 1,67 μM, *i.e.*, at concentrations c. 230-fold lower than azathioprine (Table 2.2). In contrast to azathioprine, 6-MP-riboside also showed weak antimicrobial activity, being able to inhibit bacterial growth at 64μg/ml corresponding to 214 μM (Table 2.2).

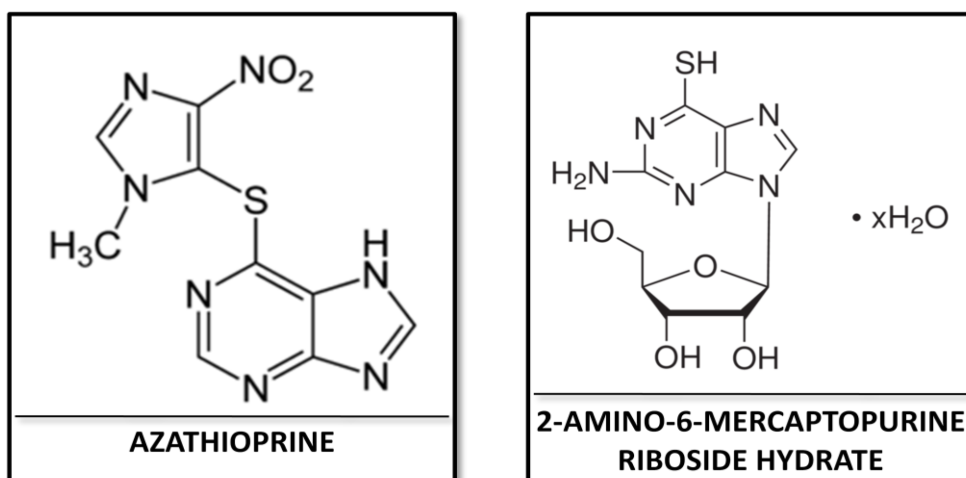


Figure 2.6 Chemical of purine analogues azathioprine and 2-amino-6-mercaptopurine riboside hydrate

Assays	Inhibition by azathioprine (μM)	Inhibition by 6-MP-riboside (μM)
Biofilm formation[A] (crystal violet assay)	IC_{50}^{*} = 383 ± 67	IC_{50}^{*} = 1,67 ± 0,22
c-di-GMP biosynthesis (HPLC)[B]	IC_{50}^{*} = 180	N.D.
Bacterial growth inhibition[C]	>923	214 ± 28

Table 2.2 IC50* =azathioprine/6-MP-riboside concentration inhibiting the reaction by 50%

[A]Average of three experiments: control values for PHL565W/pTOPOWspR = 35.7, for PHL565/pTOPO = 0,39 adhesion units

[B]Average of two determinations with very similar values: control values PHL565W/pTOPOWspR = 30.7nmol/g dry weight.

[C]Average of three independent experiments. Values determined by visual inspection.

N.D = not detemined

Finally we decided to observe the effects of azathioprine, 6-MP and sulfathiazole on *csgBAC* transcription levels in PHL856/pTOPOWspR. As shown in Figure 2.7, both azathioprine and 6-MP-riboside (red and green line respectively) strongly inhibited *csgBAC* WspR-dependent transcription. In particular 6-MP-riboside is active at lower concentrations than azathioprine.

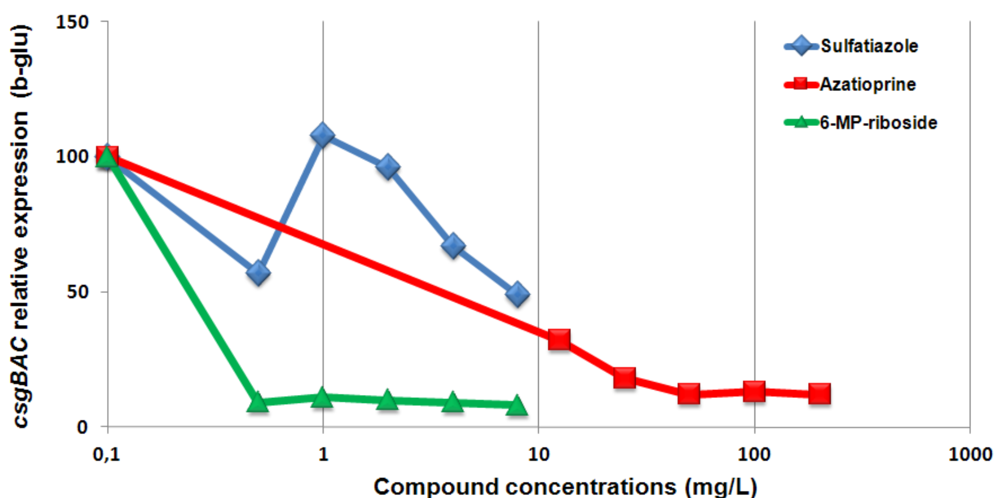


Figure 2.7 Dose-dependent inhibition of relative *csgBAC* expression in presence of sulfathiazole (blue line), azathioprine (red line), 6-MP-riboside (green line) in PHL856/pTOPOWspR. Results obtained from overnight cultures grown in M9 Glu-sup medium at 30°C Results are the average of four different experiments; standard deviation was lower than 5%.

In PHL856/pTOPOWspR, sulfathiazole inhibited *csgBAC* transcription by more than 50% at 8 μg/ml, corresponding to 31,2μM. Curiously, sulfathiazole showed a biphasic inhibition curve characterized by partial inhibition at lower concentrations, followed by an increase of *csgBAC* transcription at intermediate concentrations and then by a secondary inhibition curve (Figure 2.7). This biphasic trend might be due to multiple effects on physiological signals affecting curli expression.

Since we observed promising inhibition of WspR-dependent *csgBAC* transcription by azathioprine and 6-MP-riboside, we tested if these two compounds like sulfathiazole negatively affected the expression of *csgBAC* operon in AM70/pTOPOYdaM. As shown in Figure 2.8 both azathioprine and 6-MP-riboside failed to inhibit YdaM-dependent *csgBAC* transcription; treatment with azathioprine even seems stimulate *csgBAC* transcription (Figure 2.8). These observation was only active in the screening using WspR would suggest a specificity of action against the WspR protein of Azathioprine and 6-MP.

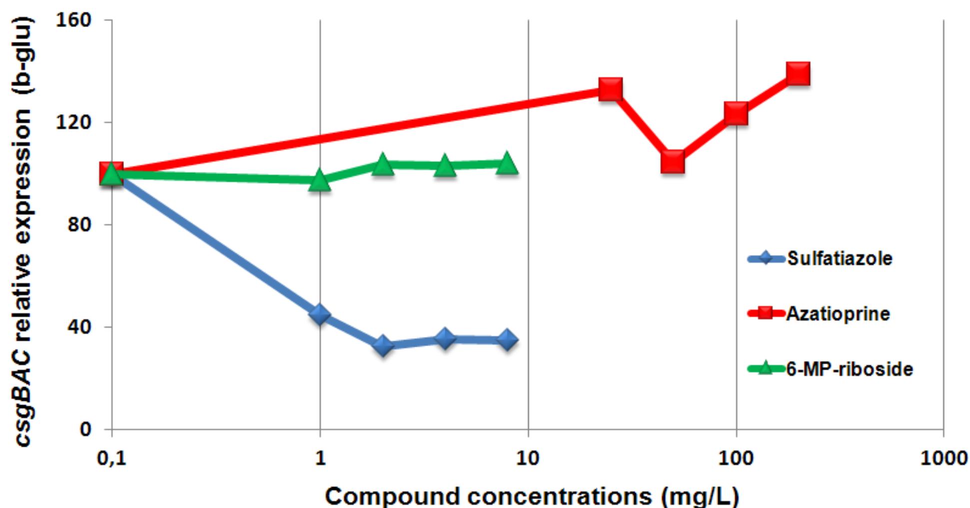


Figure 2.8 Dose-dependent inhibition of relative *csgBAC* expression in presence of sulfathiazole (blue line), azathioprine (red line), 6-MP-riboside (green line) in PHL856/pTOPOYdaM. Results obtained from overnight cultures grown in M9 Glu-sup medium at 30°C. Results are the average of four different experiments; standard deviation was lower than 7%.

2.2.7 SULFATHIAZOLE AND AZATHIOPRINE PREVENT BIOFILM FORMATION IN CLINICAL ISOLATES

Since sulfathiazole, azathioprine and 6-MP showed some ability as biofilm inhibitors we tested their spectrum of action on uropathogenic clinical isolates of *E. coli* collected from urinary catheters. We tested the behaviour of 26 clinical isolates belonging to different bacterial species (*e.g. P. aeruginosa* , *E. coli, Proteus mirabilis*) on Congo Red (CR), CalcoFluor (CF) and in the biofilm formation (crystal violet - CV) assay. Among these isolates, eight strains were able to produce EPS and to form biofilm, two of which (*E .coli* isolates 16 and 74) were able to produce huge amounts of EPSs as judged by CF test and to form a thick biofilm in microtiter plate (Figure 2.9). Thus we tested sulfathiazole, azathioprine and 6-MP-riboside and found that all three compounds were active in preventing EPSs production and biofilm formation in *E. coli* 16 and *E. coli* 74. As shown in Figure 2.9, when sulfathiazole was added to the growth medium (at a concentration of 50μg/ml corresponding to 232μM), it inhibited both EPSs production and biofilm formation. Similar results were obtained when we treated the two strains with azathioprine and 6-MP-riboside (data not shown).

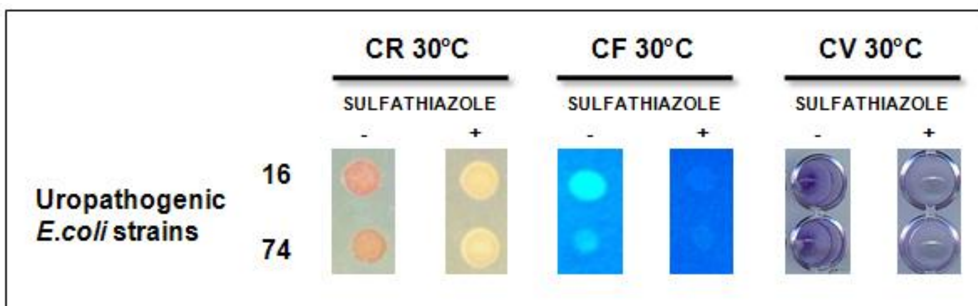


Figure 2.9 Effects of sulfathiazole on clinical isolates of *E. coli.* At a concentration of 50µg/ml (corresponding to 232µM) sulfathiazole prevents EPS production and biofilm formation. Semi-quantitative evaluation of biofilm in crystal violet assays gave adhesion values of 8,18 for *E. coli* 16, 6,58 *E. coli* 74and 0,88 and 0,56 for *E.coli* 16 and 74 treated with sulfathiazole respectively.

We tested the three compounds also on clinical isolates other than *E. coli* but no remarkable effects were detected (data not shown).

2.3 DISCUSSION

In this chapter, I have described a novel screening strategy for inhibitors of DGCs, a class of bacterial enzymes responsible for the biosynthesis of the signal molecule c-di-GMP. c-di-GMP-mediated biofilm formation is an important factor in host colonization and appears to play a major role in chronic diseases such as lung infection in cystic fibrosis patients (Häussler 2004; Kulasakara *et al.* 2006; Tamayo *et al.* 2007). Thus, inhibition of enzymes involved in c-di-GMP biosynthesis might counteract host colonization by pathogenic bacteria and complement antimicrobial therapies with conventional antibiotics. The screening strategy described in this report takes advantage of well defined genetic systems (Zogaj *et al.* 2001; Simm et al. 2004) and uses simple and established assays performed on living bacteria to identify DGC inhibitors able to cross the bacterial membrane. Although the assays used in the initial steps of our screening strategy do not directly detect intracellular c-di-GMP concentrations, they can measure DGC-dependent EPS production and biofilm formation (CR, CF and crystal violet assays, Figure 2.2 and 2.4) and DGC-dependent activation of gene expression (β-glucuronidase reporter gene assays, Figure 2.3 and Figure 2.5). The possibility of utilizing three different DGCs (AdrA, YdaM and WspR) from

different bacterial species in β-glucuronidase assay is an additional asset, since our aim is to identify molecules active on more than one specific DGC.

The potential of the proposed approach was validated by a screening of a commercially available library of chemical compounds with known biological activities, a screening approach known as Selective Optimization of Side Activities (Wermuth 2006). In two different round of screening out of the ca. 1,120 compounds tested (each round), we found that two molecule, the antimicrobial agent sulfathiazole, and the purine analogue immunosuppressive drug azathioprine resulted in discoloration of red phenotype on CR medium. No other molecule with antimicrobial activity present in the Prestwick chemical library (*e.g.*, amikacin, tobramycin, dirithromycin, pipemedic acid, and ofloxacin) showed any effect on CR phenotype of our reporter strains at concentrations allowing bacterial growth, suggesting that the effect of sulfathiazole or azathioprine are specific and not due to partial growth inhibition. Thus, sulfathiazole, azathioprine and its metabolite 6-MP-riboside were further investigated in the secondary screening assays: the three compounds displayed at subinhibitory concentrations anti-biofilm activity and were able to prevent DGC-mediated activation of the *csgA* gene (Table 2.1, Table 2.2 and Figure 2.7). In addition sulfathiazole and azathioprine were able to prevent c-di-GMP biosynthesis in bacterial cells (Table 2.1 and Table 2.2). However despite their inhibitory activity on c-di-GMP synthesis *in vivo* neither sulfathiazole nor azathioprine showed any direct interaction with purified DGC from *C. crescentus* PleD in any *in vitro* DGC assay performed by the research group of Prof. Francesca Cutruzzolà (Sapienza University of Roma, Rome, Italy). Enzymatic assays using WspR and YdaM are planned, but the purification of these two DGCs has been not carried out yet due to its technical difficulties.

Interestingly sulfahiazole, azathioprine and 6-MP-riboside are all known inhibitors of nucleotide biosynthesis. Sulfathiazole belongs to the sulfonamide class of antimicrobials and is an inhibitor of di- and tetrahydrofolate biosynthesis via interaction with the dihydropteroate synthase FolP (Vedantam and Nichols 1998; Haasum *et al.* 2001); depletion of intracellular tetrahydrofolate in turn affects various metabolic pathways, including biosynthesis of purine nucleotides. Azathioprine is used as an immunosuppressive an anti-inflammatory drug (Bradford and Shih 2011; Meurer *et al.* 2012) and is reportedly a pro-drug being metabolized to 6-MP. However, it has been reported that azathioprine inhibits 5-aminoimidazole-4-carboxamide ribotide transformylase (AICAR transformylase) *in vitro* (Ha *et al.* 1990). AICAR transformylase is widely conserved, and in bacteria it is encoded by the *purH* gene. PurH catalyzes the last two steps of *de novo* purine biosynthesis (Flannigan *et*

al. 1990; Qiu *et al.* 2011) thus, like sulfathiazole, azathioprine might also affect nucleotide biosynthesis in bacteria. It has been reported that, mutations in nucleotide biosynthetic genes can impair biofilm formation and surface adhesion in both *P. aeruginosa* (Ueda *et al.* 2009) and in *E. coli* (Garavaglia *et al.* 2012) strongly suggesting that perturbation of intracellular nucleotide pools could indeed interfere with molecular signaling leading to biofilm formation. These results seem to suggest that inhibition of c-di-GMP biosynthesis by sulfathiazole, azathioprine and 6-MP-riboside might take place in an indirect fashion, namely through inhibition of the intracellular nucleotide pool; depletion of the intracellular levels of GTP, the substrate of DGC enzymes, would in turn affect their activity. However, sulfathiazole, azathioprine and 6-MP are promising anti-biofilm compounds; since they showed, a clear inhibition of both in *E. coli* laboratory strain and in clinical isolates (Figure 2.9). These results further confirm the effectiveness of our screening system, which could be used to screen larger chemical libraries in order to identify new compounds able to affect DGC activity or biofilm formation

2.4 MATERIALS AND METHODS

2.4.1 BACTERIAL STRAINS AND GROWTH CONDITIONS

Bacterial strains used in this work are listed in Table 2.3. Bacteria were grown at 30°C, a temperature facilitating EPS production (in particular cellulose) and biofilm formation in *Enterobacteria* (Zogaj *et al.* 2001; Robbe-Saule *et al.* 2006; Gualdi *et al.* 2008) in M9 salts (Na_2HPO_4 33.9 g/L, KH_2PO_4 15 g/L, NaCl 2.5 g/L and NH_4Cl 5 g/L) supplemented with 0.5% (w/v) glucose, 0.02% peptone, and 0.01% yeast extract (M9 Glu-sup medium; Brombacher *et al.* 2006). When needed, antibiotics were used at the following concentrations: ampicillin, 100 μg mL^{-1}; chloramphenicol, 35 μg mL^{-1}; and kanamycin, 50 μg mL^{-1}. For growth on Congo Red-supplemented or Calcofluor-supplemented agar media, bacteria were inoculated in M9 Glu-sup medium in a microtiter plate, and the cultures were spotted, using a replicator, on CR medium (1% Casamino acids, 0.15% yeast extract, 0.005% $MgSO_4$, 0.0005% $MnCl_2$, 2% agar) to which 0.004% CR and 0.002% Coomassie blue [for

Congo Red (CR) medium] or 0.005% Calcofluor [for Calcofluor (CF) medium] were added after autoclaving. Bacteria were grown for 18– 20 h at 30°C; staining was better detected after 24-48 h of additional incubation at 4°C

.

Table 2.3 Bacterial strain and plasmids used in this study.

	Relevant genotype or characteristics	**Reference or source**
***E. coli* strains**		
MG1655	Standard reference strain F^-, λ^-, rph-1	Blattner *et al.* 1997
PHL856	MG1655 *csgA::uidA-kan*	Gualdi *et al.* 2008
AM70	MG1655 *ΔcsgA::cat*	This thesis
AM73	MG1655 *ΔcsgA::cat, ΔbcsABZC::kan*	This thesis
PHL565W	MG1655 derivated with an attenuated expression of the *rpoS* gene encoding σ^S due to an unknown mutation. W= white on Congo Red. Formerly called PHL565.	Gualdi *et al.* 2007
16	Multidrug resistant clinical isolate collected from a urinary catheter	Pio Albergo Trivulzio hospital - Milan
74	Multidrug resistant clinical isolate collected from a urinary catheter	Pio Albergo Trivulzio hospital - Milan
***P. areuginosa* strain**		
PAO1	Standard reference strain	Stover *et al.* 2000
Plasmids		
pTOPO	Control vector allowing direct cloning of PCR products, ampicillin and kanamycin resistance	Invitrogen
pTOPOAdrA$_{wt}$	*adrA* gene cloned as PCR product into pTOPO vector	Gualdi *et al.* 2008
pTOPOAdrA$_{GGAAF}$	*adrA* allele carrying mutation resulting in GGDEF→GGAAF change in AdrA protein catalytic site	This thesis
pTOPOYdaM	*ydaM* gene cloned as PCR product into pTOPO vector	This thesis
pTOPOWspR	*wspR* gene cloned as PCR product into pTOPO vector	This thesis

Table 2.4 Primers used in this study.

Primers	Sequence	Utilization
adrA_fwr	5'-GCTCCGTCTCTATAATTTGGG-3'	Construction of pTOPOAdrA$_{wt}$ and *adrA* mutant
adrA_rev	5'-ATCCTGATGACTTTCGCCGG-3'	Construction of pTOPOAdrA$_{wt}$ and *adrA* mutant
adrA-mut_fwr	5'-CTGCGCGCTAGCGATGTGATTGGTCGGTTT GGCGGCGCTGCGTTTG-3'	Construction *adrA* mutant
adrA-mut_rev	5'-CAATCACATCG**C**TAGCGCGCAG-3'	Construction *adrA* mutant
ydaM_fwr	5'-GCGATCGGATAGCAACAA-3'	*ydaM* cloning
ydaM_rev	5'-GAAGTCGTTGATCTCGAC-3'	*ydaM* cloning
wspR_fwr	5'-GGTCCCGGAGAGAAAC-3'	*wspR* cloning
wspR_rev	5'-GCCGGCCTCTATTTAATGC-3'	*wspR* cloning
csgA_cam_fwr	5'-TTTCCATTCGACTTTTAAATCAATCCGAT GGGGGTTTTACTACCTGTGACGGAAGATCA-3'	*csgA* inactivation
csgA_cam_rev	5′-AACAGGGCTTGCGCCCTGTTTCTGTAATAC AAATGATGTAGGGCACCAATAACTGCCTT-3'	*csgA* inactivation
cat_rev	5′-GGGCACCAATAACTGCCTTA-3′	Mutant verification
csgA_fwr	5′-ACAGTCGCAAATGGCTATTC-3	Mutant verification

2.4.2 BIOFILM FORMATION ASSAYS

Biofilm formation in microtiter plates was determined by the crystal violet staining assay (O'Toole and Kolter 1998; Dorel *et al.* 1999). Bacteria were grown overnight (ca. 18 h) in liquid M9 Glu-sup medium at 30°C in polystyrene microtiter plates (0.2 mL); the liquid culture was removed, and cell density of planktonic bacteria was determined spectrophotometrically (OD_{600nm}). Cells attached to the microtiter plates were washed gently with water and stained for 20 min with 1% crystal violet, thoroughly washed with water, and dried. For semiquantitative determination of biofilms, crystal violet stained cells were resuspended in 0.2 mL of 95% ethanol by vigorous pipetting. The OD_{600nm} of crystal violet-stained biofilm cells was determined and normalized to the OD_{600nm} of the planktonic cells from the corresponding liquid cultures; this value is defined as "adhesion units".

2.4.3 PLASMID CONSTRUCTION

Plasmids and primers used in this work are respectively listed in Table 2.3 and Table 2.4. For overproduction of the AdrA,YdaM and WspR proteins, the corresponding genes were amplified by polymerase chain reaction (PCR) from the *E. coli* MG1655 (AdrA and YdaM) or *P. areuginosa* PAO1 (WspR) chromosome, and the resulting products were cloned into

the pTOPO vector. The pTOPOAdrA$_{GGAAF}$ plasmid, carrying a mutated allele of the adrA gene in which the DGC catalytic site is inactivated, was constructed as follows: the 5′ and the 3′ portions of the adrA gene were amplified by PCR using the adrA_fwr and adrA-mut_rev primers (for the 5′-portion of adrA) or the adrA-mut_fwr and the adrA_rev primers (for the 3′-portion of adrA), resulting in the following sub stitutions: G→C at nucleotide 842 of the adrA gene (creation of an NheI restriction site), C→A at nucleotide 872, and C→A at nucleotide 875. The last two mutations result in the substitution of both the aspartic and the glutamic acid residues at position 291–292 of the AdrA protein to alanine residues (GGDEF→GGAAF). Both the 5′ and the 3′ portions of the mutated adrA gene were cloned into pTOPO, and the full-length adrA gene carrying the GGAAF mutation was reconstituted by subcloning the 3′ portion of adrA into pTOPO carrying the 5′ portion of the gene, using the newly created NheI restriction site in the mutated adrA gene and the XbaI site present in the pTOPO multiple cloning site. Both the wild type and mutant alleles of the adrA gene were verified by sequencing.

2.4.4 DETERMINATION OF INTRACELLULAR c-di-GMP CONCENTRATION

Overnight cultures were collected by centrifugation, and the supernatant were carefully removed. Bacterial cells were resuspended in 0.4 M $HClO_4$ at a ratio of 45 mg cells/ 0.35 mL and broken by sonication; cell debris was removed by centrifugation (10,000×g, 10 min, 4°C). Supernatants were neutralized with 0.16 M K_2CO_3, kept on ice for 10 min, and centrifuged at 12,000×g for 3 min. Supernatants were filtered and injected into an HPLC system equipped with a diode-array detector. HPLC separation was essentially performed as described in Stocchi *et al.* (1985). A 12.5-cm Supelcosil LC-18-DB, 3 μm particle size, reversed phase column was used, and the temperature was fixed at 18°C. Elution conditions were 9 min at 100% buffer A (100 mM potassium phosphate buffer, pH 6.0), followed by step elution to 12%, 45%, and 100% buffer B (buffer A containing 20% methanol), at a flow rate of 1.3 mL/min. Purity index of c-di-GMP peak is 0.96. Its identity as genuine c-di-GMP was determined by coelution and identical UV absorption spectra with a c-di-GMP standard (purchased from Biolog, Bremen, Germany). c-di-GMP concentration was calculated based on an extinction coefficient (ε) of 23,700 at 254 nm (Hayakawa *et al.* 2003).

2.4.5 OTHER METHODS

The *E. coli* MG1655 derivative deleted in the *csgA* gene (AM70, *ΔcsgA::cam*) was constructed using the λ Red technique (Datsenko and Wanner 2000). Target gene disruption was confirmed by PCR. P1 transduction of the *ΔbcsABZC::kan* mutation (Da Re and Ghigo 2006) was carried out as described (Miller 1972). β-Glucuronidase specific activity was measured by hydrolysis of p-nitrophenyl-β-D-glucuronide into p-nitrophenol at 405 nm (Bardonnet and Blanco 1992). Antimicrobial activity was determined as the minimal inhibitory concentration (MIC) in liquid M9 Glu-sup medium, using the broth microdilution method according to the guidelines of the Clinical and Laboratory Standards Institute (2006). Inhibition of bacterial growth was determined by lack of turbidity by visual inspection. Inhibition of biofilm formation was performed on the same samples by the crystal violet assay (described in section 2.4.2).

CHAPTER III

CURLI AND POLY-*N*-ACETYLGLUCOSAMINE PRODUCTION ARE CONTROLLED BY YddV-Dos COMPLEX

Results described in this chapter have been published in the following publications:

Tagliabue L., Maciąg A., Antoniani D. and Landini P. (2010). The *yddV-dos* operon controls biofilm formation through the regulation of genes encoding curli fibers' subunits in aerobically growing *Escherichia coli*. *FEMS Immunol. Med. Microbiol*. 59:477-484.
(http://onlinelibrary.wiley.com/resolve/openurl?genre=article&sid=nlm:pubmed&issn=0928-8244&date=2010&volume=59&issue=3&spage=477)

Tagliabue L., Antoniani D., Maciąg A., Bocci P., Raffaelli N. and Landini P. (2010). The diguanylate cyclase YddV controls production of the exopolysaccharide poly-*N*-acetylglucosamine (PNAG) through regulation of the PNAG biosynthetic *pgaABCD* operon. *Microbiol*. 156: 2901 - 2911.
(http://mic.sgmjournals.org/cgi/pmidlookup?view=long&pmid=20576684).

3.1 INTRODUCTION

Most bacteria are able to switch between two different 'lifestyles': single cells (planktonic mode) and biofilm, *i.e.*, a sessile microbial community. In particular, biofilm cells are characterized by production of adhesion factors and extracellular polysaccharides (EPS), resistance to environmental stresses, and lower sensitivity to antibiotics compared with planktonic cells (Costerton *et al.* 1995; Anderl *et al.* 2000). The transition from planktonic cells to biofilm is regulated by environmental and physiological cues, relayed to the bacterial cell by signal molecules such as cyclic di-GMP (c-di-GMP). Intracellular levels of c-di-GMP are regulated by two classes of enzymes: diguanylate cyclases (DGCs, c-di-GMP biosynthetic enzymes), also termed GGDEF proteins from the conserved amino acid sequence in their catalytic site, and c-di-GMP phosphodiesterases (PDEs), which degrade c-di-GMP (Cotter and Stibitz 2007). While in Gram negative bacteria genes encoding DGC and PDE proteins are present in high numbers, they are much less conserved in Gram positive bacteria (Galperin 2004), where c-di-GMP does not appear to play a significant role in biofilm-related cell processes (Holland *et al.* 2008). The high number of DGC- and PDE-encoding genes in Gram negative bacteria would suggest that c-di-GMP biosynthesis and degradation constitute a mechanism for signal transduction involving the interaction of c-di-GMP-responsive proteins with specific DGCs. In this chapter I will described how *yddV-dos* operon affects the production of adhesion factors in *E. coli*. In particular the

yddV-dos operon encodes, respectively, a protein with DGC activity (which synthesize c-di-GMP) and a PDE that can degrade c-di-GMP to pGpG (the linear form of diguanylic acid), not known to function as a signal molecule (Schmidt *et al.* 2005). Dos stands for direct oxygen sensor, because the Dos protein is complexed to a heme prosthetic group that can bind O_2, CO and nitric oxide (NO) (Delgado-Nixon *et al.* 2000). A recent publication (Tuckerman *et al.* 2009) has reported that YddV is also a heme-binding oxygen sensor, and that YddV and Dos interact to form a stable protein complex. Although it has been reported that YddV overexpression can stimulate biofilm formation (Méndez-Ortiz *et al.* 2006), the targets of *yddV*-dependent biofilm induction have not yet been identified. In this chapter, I will discuss the role of the *yddV-dos* operon in the regulation of both curli and poly-*N*-acetylglucosamine (PNAG) production, two of main adhesion factor of *E. coli*.

3.2 RESULTS

3.2.1 PARTIAL DELETION OF THE *yddV* AND *dos* GENES

The *yddV-dos* operon is arguably the most strongly expressed c-di-GMP related operon in *E. coli* (Pesavento *et al*. 2008; Sommerfeldt *et al.* 2009). Overexpression of the YddV protein was reported to stimulate biofilm formation and to impair cell motility (Méndez-Ortiz *et al.* 2006), consistent with YddV DGC activity. However, it is not clear which adhesion factors mediate YddV-dependent biofilm formation. In order to evaluate more precisely the contribution of c-di-GMP synthesis and turnover toward YddV and Dos protein activities, our mutagenesis strategy targeted exclusively the region of the gene encoding the domains involved in c-di-GMP metabolism, allowing the production of truncated YddV and Dos proteins carrying functional hemebinding and sensor domains. Because *yddV* and *dos* are part of the same transcriptional unit (Méndez-Ortiz *et al.* 2006), insertions of antibiotic resistance cassettes into the *yddV* gene can result in transcription termination, thus preventing *dos* transcription. However, in the AM95 (*yddV*$\Delta_{931–1383}$::cat) mutant, replacement of the distal part of the *yddV* gene by the chloramphenicol acetyl-transferase (*cat*) gene, placed in the same orientation, results in semi-constitutive transcription of the *dos* gene from the *cat* promoter, as determined by quantitative real-time PCR (qRT-PCR; data not shown). Because YddV and Dos constitute a highly expressed protein complex possessing both DGC and PDE activity (Sommerfeldt *et al.* 2009; Tuckerman *et al.* 2009), the production of truncated forms of either YddV or Dos should result in the formation of mutant YddV–Dos protein complexes unbalanced either towards accumulation or towards degradation of c-di-GMP.

However, we found that mutants in the *dos* gene showed phenotypic instability at the level of cell aggregation in liquid culture and Congo red binding, suggesting that the *dos* mutant strain might accumulate spontaneous mutations suppressing the *dos* defect. Thus, the *dos* mutant strain was not investigated any further, and we focused on the *yddV* mutant AM95 and on MG1655 derivatives overexpressing either the YddV or the Dos proteins from multicopy plasmids.

3.2.2 EFFECTS OF THE *yddV* AND *dos* MUTATIONS ON CONGO RED BINDING AND BIOFILM FORMATION

To determine the possible effects of mutations in the *yddV* gene on curli production, we performed Congo red-binding assays using CR medium (for medium composition see section 3.4.1). Curli fibers bind Congo red with very high affinity, due to their β-amyloid structure (Olsén *et al.* 1989; Chapman *et al.* 2002). Congo red can bind, albeit with a lower affinity, other cell surface-exposed structures, such as the EPSs cellulose and poly-*N*-acetylglucosamine (Jones *et al.* 1999; Zogaj *et al.* 2001); however, in *E. coli* MG1655, due to the low production of extracellular polysaccharides, the red phenotype on CR medium is totally dependent on curli production (Gualdi *et al.* 2008). Indeed, a mutant carrying a null mutation in the *csgA* gene, encoding the main curli structural subunit, displays a white phenotype on CR medium (Figure 3.1A see also Figure 2.2A Chapter II). The *yddV*$\Delta_{931-1383}$*::cat* mutation resulted in a clear, albeit partial, loss of the red phenotype on CR medium, indicative of a reduction in curli production. To further confirm the effects of the mutation in the *yddV* gene, we cloned either the *yddV* or the *dos* genes into the pGEM-T Easy vector, under the control of the *lac* promoter, producing the pGEM-YddV$_{WT}$ and pGEM-Dos$_{WT}$ plasmids (Table 3.4 see section 3.4). In addition, we constructed plasmids carrying mutant alleles of either gene (pGEM-YddV$_{GGAAF}$ and pGEM-Dos$_{AAA}$, Table 3.4 see section 3.4), in which the coding sequence for the amino acids responsible for either DGC activity (in the YddV protein) or PDE activity (in the Dos protein) had been altered. The substitution of GGDEF motif into the DGC catalytic site to GGAAF results in a drastic loss (>90%) of DGC activity (De *et al.* 2008; see also Chapter II section 2.2.2). In the Dos protein, the glutamic acid and leucine in the EAL motif were changed to alanine residues, giving rise to the Dos$_{AAA}$ mutant; mutations affecting the EAL motif abolish PDE activity (Kirillina *et al.* 2004; Bassis and Visick 2010). Transformation of the *yddV* mutant AM95 strain with pGEM-YddV$_{WT}$, but not with pGEM-YddV$_{GGAAF}$, restored the red phenotype on CR medium (Figure 3.1B), indicating that YddV can affect the CR phenotype in a manner

dependent on its DGC activity. Transformation of MG1655 with the pGEM-Dos_{WT} plasmid (Figure 3.1C) resulted in a white CR phenotype, consistent with a negative role of Dos in curli production. In contrast, no effects were observed on the CR phenotype in the MG1655 strain harboring the pGEM-Dos_{AAA} plasmid, carrying the mutant Dos protein impaired in its PDE activity.

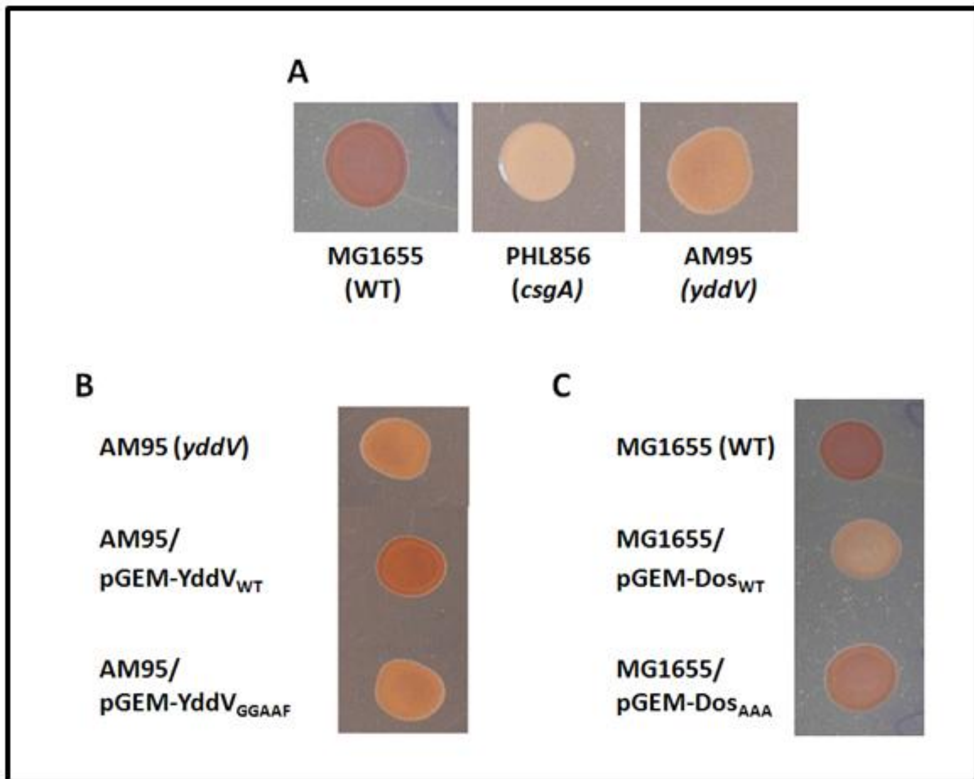


Figure 3.1 **A** Congo red phenotype of MG1655 (WT), PHL856 (*csgA*) and AM95 (*yddV*). **B** AM95 (*yddV*) strain transformed with either pGEM-$YddV_{WT}$ or pGEMYddV_{GGAAF}. **C** MG1655 strain transformed with either pGEM-Dos_{WT} or pGEM-Dos_{AAA}.

In *E. coli* MG1655, curli fibers are the main determinant for adhesion to abiotic surfaces (Prigent-Combaret *et al*. 2000). Thus, we confirmed the results of Congo red-binding assays by biofilm formation experiments on polystyrene microtiter plates (Figure 3.2). Consistent with the pivotal role of curli in adhesion to abiotic surfaces, biofilm formation on microtiter plates was reduced by about 10-fold by the inactivation of the *csgA* gene, encoding the major curli subunit (Figure 3.2), as well as by growth at 37 °C (data not shown), the temperature at which curli fibers are not produced in most enterobacteria (Römling *et al.* 1998a).

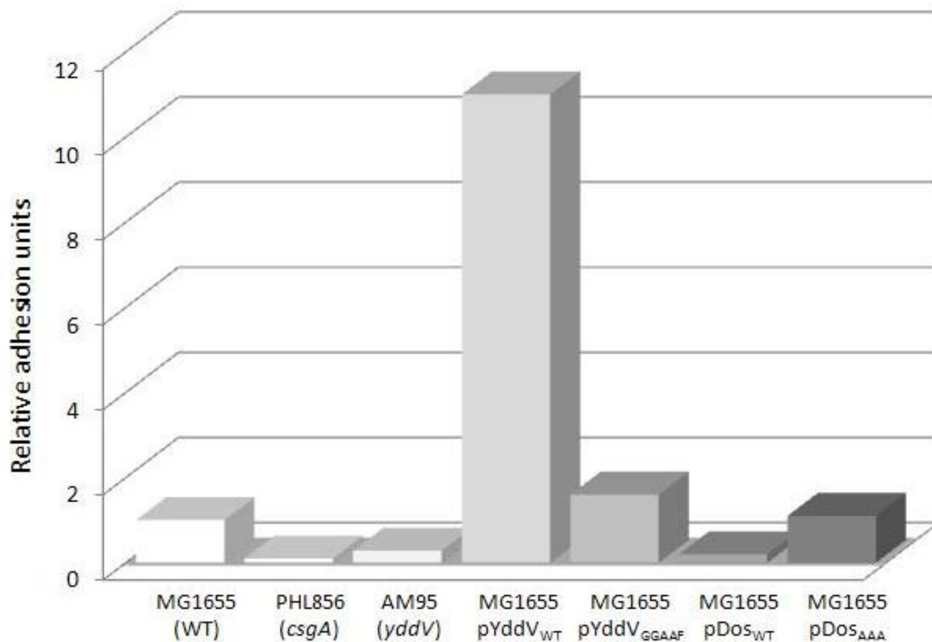


Figure 3.2 Surface adhesion on polystyrene microtiter plates by strains MG1655 (WT), PHL856 (*csgA*), AM95 (*yddV*), and MG1655 transformed with pGEM-YddV$_{WT}$, pGEM-YddV$_{GGAAF}$, pGEM-Dos$_{WT}$ and pGEM-Dos$_{AAA}$. The relative adhesion value was set to 1 for MG1655; the actual adhesion unit for MG1655 was 3.1. Results are the average of three independent experiments, with standard deviations always lower than 10%.

Inactivation of the *yddV* gene resulted in a c. 3.5-fold reduction in biofilm formation. Overexpression of YddV$_{WT}$, but not of the YddV$_{GGAAF}$ protein, results in strong biofilm stimulation (Figure 3.2), in agreement with CR phenotypes (Figure 3.1). Overexpression of the Dos protein mimicked the effects of the *yddV* mutation, resulting in decreased biofilm production; however, no effect was detected for overexpression of the Dos mutant protein impaired in PDE activity (Figure 3.2). Thus, the results of Congo red binding studies and biofilm formation experiments strongly support the hypothesis that the YddV and Dos proteins control curli production through the modulation of intracellular c-di-GMP concentrations.

3.2.3 EFFECTS OF THE *yddV* AND *dos* MUTATIONS ON CURLI GENE EXPRESSION

The regulation of adhesion factors production by DGCs can take place at different levels, such as allosteric activation, as in the stimulation of cellulose biosynthesis by AdrA (Zogaj *et al.* 2001), or gene regulation, such as in the transcription regulation of the *csgDEFG* operon

by YdaM and YegE (Sommerfeldt *et al.* 2009). We tested the possibility that the *yddV* gene might affect the CR phenotype and adhesion to polystyrene through gene expression regulation of the curli-encoding operons. Curli production and assembly is mediated by two divergent operons; *csgDEFG* encodes the transport and assembly proteins and the CsgD regulator, which in turn activates the *csgBAC* operon, encoding curli structural subunits (Römling *et al.* 1998b). Since curli genes are subject to growth phase-dependent regulation mediated by the *rpoS* gene (Römling *et al.* 1998b), we assessed the effects of the *yddV* mutation at different growth stages: early exponential phase (OD_{600nm}=0.25), late exponential phase (OD_{600nm}=0.7) and stationary phase (overnight cultures, $OD_{600nm}\geq 2.5$). Transcription levels of the *csgB* and *csgD* genes in M9 Glu-sup medium at 30 °C were determined by qRT-PCR (Table 3.1). Interestingly, the expression of *csgD* and *csgB* follows different kinetics: while *csgB* is only induced in the late stationary phase, *csgD* transcription levels are very similar both in the exponential and in the stationary phase. A different timing between *csgD* and *csgB* transcription in *E. coli* MG1655 has already been reported (Prigent-Combaret *et al.* 2001).

Table 3.1 Relative expression of *csgB* and *csgD* genes in MG1655 vs. AM95 (*yddV::cat*).

Genes	***csgB***		***csgD***		***adrA***	
Strains	**MG1655** (WT)	**AM95** (*yddV::cat*)	**MG1655** (WT)	**AM95** (*yddV::cat*)	**MG1655** (WT)	**AM95** (*yddV::cat*)
Growth conditions						
Early exponential (OD_{600nm}=0,25)	1*	0,7	1*	0,6	ND	ND
Late exponential (OD_{600nm}=0,7)	0,8	0,9	1,5	0,7	ND	ND
Stationary (OD_{600nm} 2,5)	391	0,9	1,4	0,6	1*	0,74
Stationary anoxic (OD_{600nm} 1,6)	57,2	22,4	1,6	1,4	ND	ND

*ΔC_t between the gene of interest and the 16S gene was arbitrarily set at 1 for MG1655 in the early exponential growth phase for *csgB* and *csgD* genes, and in stationary phase for *adrA*. The actual ΔC_t values were: *csgD*=15.0; *csgB*=21.7; *adrA*= 22.4. ΔC_t between the gene of interest and the 16S gene for different growth phases and for mutant strains are expressed as relative values. Values are the average of two independent experiments performed in duplicated. ND, not determined.

Although the lack of stationary-phase-dependent-activation of the *csgD* gene might appear to be surprising, *rpoS*-dependent gene expression during the exponential phase is rather common (Dong *et al.* 2008); indeed, the expression of both *csgB* and *csgD* is totally abolished in the *rpoS*-deficient EB1.3 mutant derivative of MG1655 (data not shown). *yddV* inactivation caused a drastic decrease in *csgB* expression (c. 400-fold reduction, Table 3.1),

while showed a much more reduced effect on *csgD* transcription (c. 2.5-fold), suggesting that the YddV protein specifically regulates the transcription of the *csgBAC* operon. Overexpression of either the YddV or the Dos protein confirmed this result, showing *csgBAC* upregulation by YddV and downregulation by Dos, in a manner dependent on their DGC and PDE activities, respectively (Table 3.2). The observation that YddV regulates *csgBAC* transcription, which is also dependent on the CsgD protein, may suggest that c-di-GMP synthesis by YddV might trigger CsgD activity as a transcription regulator. To test this hypothesis, we studied the effect of the *yddV* mutation on the expression of *adrA*, a CsgD-dependent gene involved in the regulation of cellulose production (Zogaj *et al.* 2001): as shown in Table 3.1, *adrA* transcript levels were not significantly affected by *yddV* inactivation, suggesting that the CsgD protein can function as a transcription activator in the *yddV* mutant strain AM95. Both the YddV and the Dos protein require binding of their heme prosthetic groups to O_2, or alternatively to NO, in order to trigger either DGC or PDE activity (Taguchi *et al.* 2004; Tuckerman *et al.* 2009). Thus, we measured *csgB* and *csgD* expression levels in bacteria grown in oxygen limitation, comparing MG1655 with its $yddV_{\Delta 931-1383}$*::cat* mutant derivative. Growth under anoxic conditions did not affect *csgD* transcript levels, while reducing *csgB* expression by *c.* 7-fold; *yddV* inactivation resulted only in a *c.* 2.5-fold reduction in *csgB* transcript levels, vs. the *c.* 400-fold reduction in aerobic growth (Table 3.1), suggesting that YddV-dependent regulation of the *csgBAC* operon is bypassed under oxygen-limiting conditions. Consistent with this observation, no effect on *csgBAC* expression by either YddV or Dos overexpression could be detected in MG1655 grown in oxygen limitation (Table 3.2).

Table 3.2 Relative expression of *csgB* and *csgD* genes in response to either YddV or Dos overexpression

Strains	***csgB*** expression (**aerobic** conditions)	***csgB*** expression (**anoxic** conditions)	***csgD*** expression (**aerobic** conditions)
MG1655/pGEM-T	1*	0,38	1*
MG1655/pYddV$_{WT}$	31,2	0,32	2,1
MG1655/pYddV$_{GGAAF}$	2,3	0,45	1,6
MG1655/pDos$_{WT}$	0,06	0,34	ND
MG1655/pDos$_{AAA}$	1,04	0,37	ND

*ΔC_t between the gene of interest and the 16S gene was arbitrarily set at 1 for MG1655/pGEM-T under aerobic conditions. Actual ΔC_t values in MG1655/pGEM-T: *csgB*= 15.9; *csgD*=14.6. Values are the average of two independent experiments performed in duplicate.

3.2.4 GROWTH-PHASE DEPENDENT REGULATION OF THE *yddV-dos* OPERON.

Our results clearly indicate that a functional *yddV* gene is required for *csgBAC*, but not *csgDEFG*, expression (Table 3.1), suggesting that the YddV protein acts downstream of CsgD in the regulatory cascade leading to curli production. It is thus possible that the CsgD protein might activate the transcription of the *yddV-dos* operon and, in turn, YddV might trigger *csgBAC* expression in the stationary phase of growth. However, co-transcription of the *yddV* and the *dos* genes also raises the question of how the opposite activities of the YddV and Dos proteins are modulated. We investigated the possibility that the *yddV-dos* transcript might be processed in the stationary phase of growth, resulting in the accumulation of the YddV protein, with consequent activation of *csgBAC* expression. To address these questions, we determined both *yddV* and *dos* transcripts at different growth stages, and we tested the possible dependence of *yddV-dos* transcription on the CsgD protein by comparing MG1655 with its *csgD* mutant derivative AM75. In addition, because transcription of the *yddV-dos* operon is controlled by the *rpoS* gene (Weber *et al.* 2006; Sommerfeldt *et al.* 2009), which also regulates curli-encoding genes (Römling *et al.* 1998b), we also determined gene expression kinetics of the *yddV-dos* operon in the *rpoS* mutant derivative EB1.3.

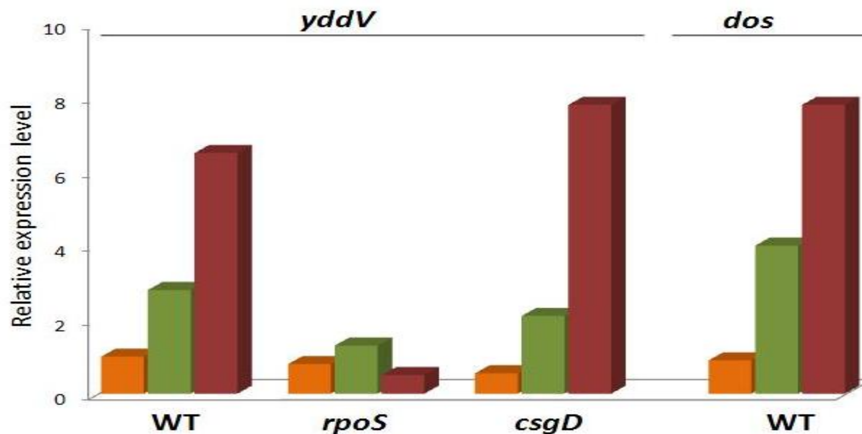


Figure 3.3 Relative expression levels of the *yddV* gene in strains MG1655 (WT), EB1.3 (*rpoS*) and AM75 (*csgD*), and of the *dos* gene in MG1655, as measured by realt-time PCR experiments. Expression values in MG1655 in the early exponential growth phase (OD_{600nm}=0.25; orange bars) (corresponding to a ΔC_t relative to 16S rRNA=16.3 for *yddV* and =15.8 for *dos*) were set to 1. The other samples were taken in late exponential phase (OD_{600nm}=0.7; green bars) and stationary phase ($OD_{600nm} \geq 2.5$; dark red bars). Data are the average of two independent experiments, each performed in duplicate.

As shown in Figure 3.3, transcription of the *yddV* gene was induced in an *rpoS*-dependent manner in the late exponential phase, reaching maximal induction in overnight cultures; in contrast, *csgD* inactivation did not affect *yddV* expression. Transcription of the *yddV* and of the *dos* genes followed a very similar pattern (Figure 3.3) and the overall ratio between *yddV* and *dos* transcripts remained constant in different growth phases, suggesting that neither *yddV* nor *dos* is subject to specific regulation at the level of mRNA processing, at least under the conditions tested.

3.2.5 OVEREXPRESSION OF DGCs

As shown in the previous sections, *yddV-dos* operon affects curli biosynthesis; thus it appears that several c-di-GMP metabolic genes are involved in curli regulation (Pesavento *et al.* 2008; Sommerfeldt *et al.* 2009). In order to investigate the effects of different DGC proteins on the production of extracellular structures and biofilm formation, we cloned DGC-encoding genes into the pGEM-T Easy multicopy plasmid, which allows constitutive expression of cloned genes in the absence of IPTG induction. In addition to the pGEM-YddV plasmid described earlier, we cloned the following DGC-encoding genes: *adrA*, encoding an activator of cellulose production (Zogaj *et al.* 2001); *ycdT*, located in the *pgaABCD* locus and co-regulated with the PNAG-biosynthetic genes (Jonas *et al.* 2008); and *ydaM*, required for expression of curli-encoding genes (Weber *et al.* 2006). Plasmid-driven expression of each of the four genes resulted in a significant increase in intracellular c-di-GMP concentrations, consistent with production of active proteins; however, while overproduction of the AdrA and the YdaM proteins resulted in a more than 150-fold increase in intracellular c-di-GMP, in agreement with previous observations (see section 2.2.2), YcdT and YddV only enhanced c-di-GMP concentration by about 10-fold (Figure 3.4). c-di-GMP intracellular concentrations did not strictly correlate with DGC overproduction levels, as judged by SDS-PAGE analysis of cell extracts (data not shown). The expression of each DGC led to a reduction in bacterial mobility (Table 3.3), in agreement with earlier observations (Méndez-Ortiz *et al.* 2006; Jonas *et al.* 2008; Pesavento *et al.* 2008).

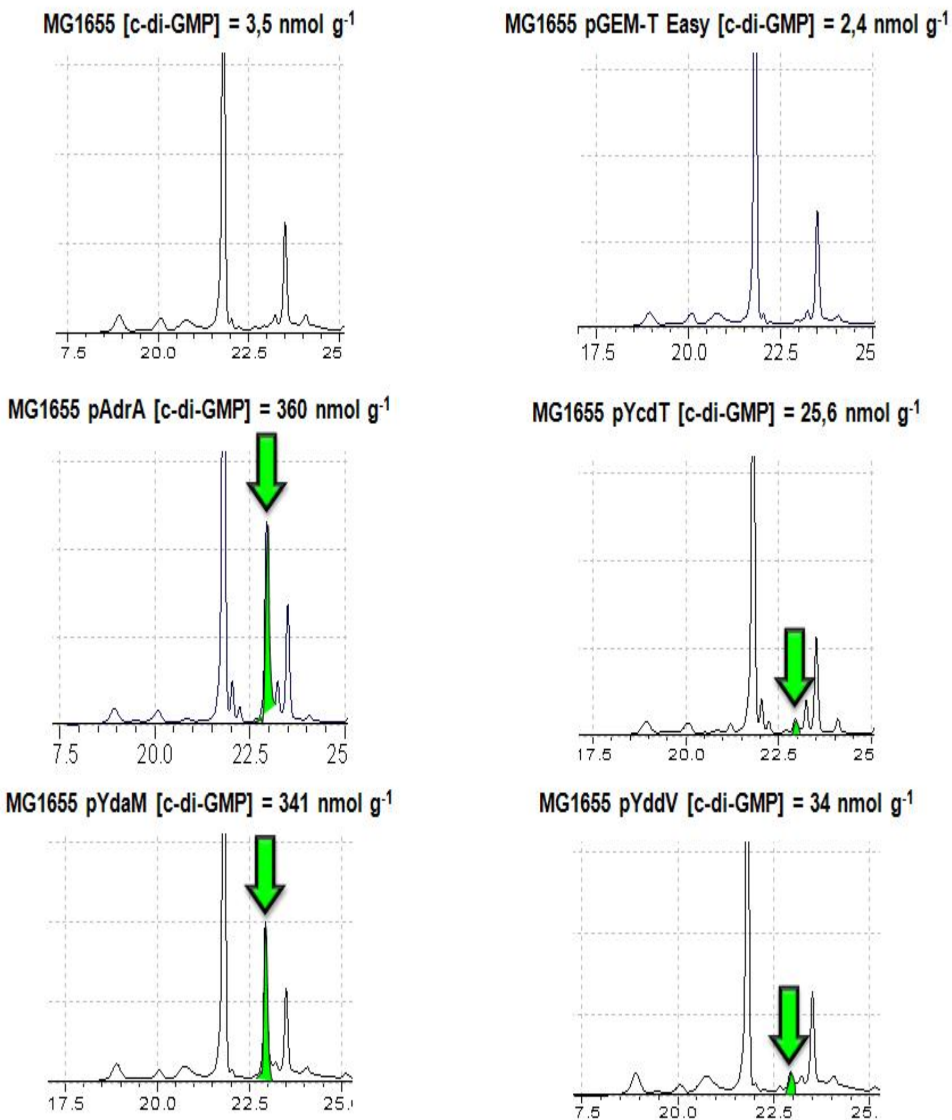


Figure 3.4 HPLC determination of intracellular c-di-GMP concentrations in MG1655 and in MG1655 transformed with the pGEM-T Easy vector or pGEM-T Easy carrying the genes encoding the DGCs AdrA, YcdT, YdaM and YddV. The green peaks marked by green arrows corresponding to c-di-GMP; the peak with a retention time of 21.8 min corresponds to NAD, while the peak at 23.5 min was not identified. The c-di-GMP concentrations ([c-di-GMP]) determined are given above each HPLC profile.

3.2.6 EFFECTS OF DGC OVEREXPRESSION ON CELL SURFACE ASSOCIATED STRUCTURES

The plasmids carrying DGC-encoding genes were used to transform a set of mutant derivatives of *E. coli* MG1655 deficient in the production of curli, cellulose or PNAG, namely: AM70 (Δ*csgA::cat*), unable to produce curli; LG26, a Δ*bcsA::kan* mutant impaired in cellulose production; AM73, a Δ*csgA*/Δ*bcsA* double mutant; and AM56, a Δ*pgaA::cat* mutant unable to export PNAG and to expose it on the cell surface (Itoh *et al.* 2008). We expected that phenotypes depending on an increased production of cell surface-associated structures caused by DGC overexpression would be abolished by inactivation of the corresponding target genes. Since curli, cellulose and PNAG affect binding of the bacterial cell surface to the dye CR (Olsén *et al.* 1989; Perry *et al.* 1990; Zogaj *et al.* 2001), we measured the effects of DGC overexpression on the color phenotype on agar medium supplemented with CR (CR medium). In the absence of DGC-overexpressing plasmids, strains carrying mutations in curli-related genes (Δ*csgA* and the Δ*csgA*/Δ*bcsA* double mutant) showed a white phenotype on CR plates (Figure 3.5). In contrast, inactivation of genes responsible for either cellulose (Δ*bcsA*) or PNAG biosynthesis (Δ*pgaA*) did not affect the red phenotype of the parental strain, consistent with previous observations that in *E. coli* MG1655, CR binding mostly depends on curli production (Gualdi *et al.* 2008; Ma and Wood 2009 see sections 2.2.2 and 3.2.2). Plasmid-driven expression of DGCs resulted in very different effects on colony phenotype on CR media: expression of the AdrA protein conferred a red phenotype upon the *csgA* mutant strain, but not upon the Δ*csgA*/Δ*bcsA* double mutant, consistent with its role as an activator of cellulose production (see section 2.2.2; Zogaj *et al.* 2001). Overexpression of YdaM did not affect the CR phenotype in MG1655 and in its Δ*pgaA* mutant derivative, but it conferred a weak red phenotype upon the curli-deficient mutant and the Δ*csgA*/Δ*bcsA* double mutant impaired in both curli and cellulose production. Since YdaM controls the production of both curli and cellulose via expression of the *csgD* gene (Weber *et al.* 2006), this observation suggests that either YdaM or CsgD triggers the production of yet additional cell surface-associated structures able to bind CR. In contrast to AdrA and YdaM, YcdT expression led to no detectable changes in CR phenotype in any of the strains tested (Figure 3.5).

However, YcdT overexpression, in addition to increasing c-di-GMP intracellular concentrations (Figure 3.5), clearly affected cell motility (Table 3.3) and colony size on LB medium (data not shown), suggesting that YcdT is produced in an active form in strains carrying the pYcdT plasmid.

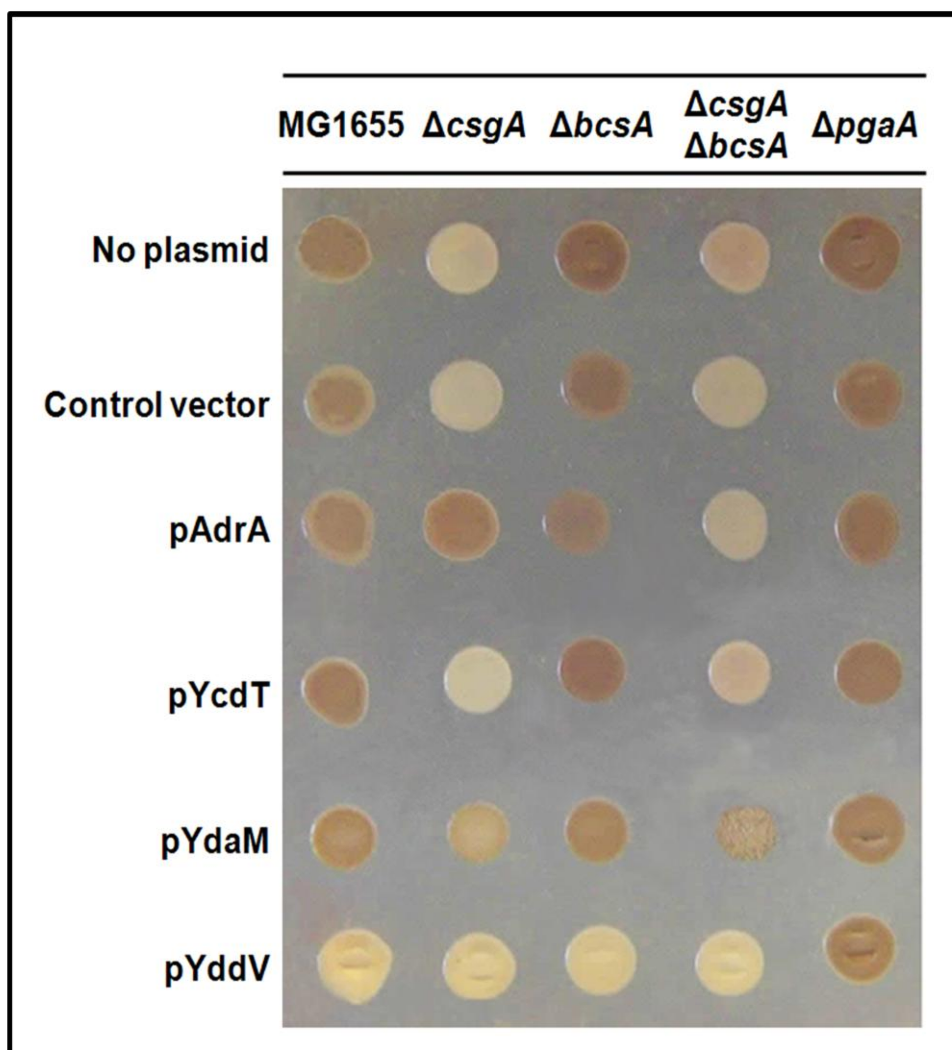


Figure 3.5 CR-binding assay. The MG1655 strain and isogenic mutants deficient in production of cell surface-associated structures were transformed with the pGEM-T Easy vector or the vector carrying the genes encoding the DGCs AdrA, YcdT, YdaM and YddV. Strains tested were: MG1655 (wild-type); Δ*csgA*, AM70 (curli-deficient mutant); Δ*bcsA*, LG26 (cellulose-deficient mutant); Δ*csgA*/Δ*bcsA*, AM73 (curli- and cellulose-deficient mutant); Δ*pgaA*, AM56 (PNAG-deficient mutant).

Table 3.3 Effects of DGCs overexpression on cell motility and cell aggregation.

Strains	Cell motility* (spot diameters mm)	Aggregation**
MG1655/pGEM-T Easy	10,5*	-
MG1655pAdrA	8	+
MG1655pYcdT	7	-
MG1655pYdaM	8,75	+++
MG1655pYddV	7	++

*) Average of two independent experiments. **) Determined by visual inspection as described in Gualdi *et al.* 2008. Results are obtained from four independent experiments.

Finally, YddV overexpression led to the loss of the red phenotype on CR medium in curli-producing strains, with the exception of the *pgaA* mutant, unable to expose PNAG on the cell surface (Figure 3.5). Although a white CR phenotype could indicate a negative regulation of curli production by YddV, the observation that the YddV-dependent white colony phenotype on CR medium requires a functional *pgaA* gene suggests that YddV overexpression triggers PNAG overproduction. Indeed, in curli-proficient strains of *E. coli*, EPS overproduction can result in the loss of the red colony phenotype on CR medium, possibly due to shielding of curli fibres (Gualdi *et al.* 2008; Ma and Wood 2009). To understand whether YddV-dependent loss of the red colony phenotype on CR medium could indeed be due to PNAG overproduction, we verified EPS production in the presence and absence of the pYddV plasmid by plating on agar medium supplemented with CalcoFluor (CF), a fluorescent dye able to bind EPS. The presence of pYddV promoted CF binding, which was however abolished in the *pgaA* mutant strain AM56, indicating that YddV overexpression increases EPS production in *pgaA*-dependent manner (Figure 3.6A). We determined YddV stimulation of surface adhesion in MG1655 and in its mutant derivatives deficient in the production of specific cell surface-associated factors. As shown in Figure 3.6 (B), YddV overexpression stimulated surface adhesion in the MG1655 strain as well as in mutants unable to synthesize either curli or cellulose, while failing to enhance biofilm formation in a *pgaA* mutant. Treatment with the PNAG-degrading enzyme Dispersin B abolished YddV-dependent stimulation of surface adhesion in MG1655 (Figure 3.6B). In contrast to YddV, overexpression of either AdrA or YcdT resulted in little or no increase in surface adhesion (Figure 3.7). Finally, YdaM overexpression stimulated PNAG production; indeed, YdaM-dependent biofilm formation was affected

(about 2-fold) by *pgaA* inactivation and by treatment with Dispersin B. However, unlike YddV, YdaM-mediated biofilm formation was totally abolished in the AM70 *csgA* mutant, indicating that it mostly depends on curli production (Figure 3.7).

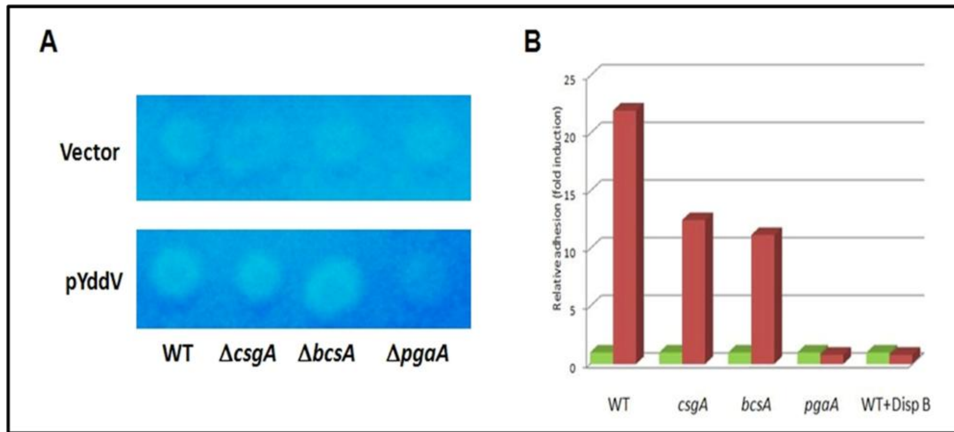


Figure 3.6 A Effects of YddV overexpression on EPS production determined by Calcofluor binding assay. The strains MG1655 (WT), AM70 (Δ*csgA*, curli-deficient mutant), LG26 (Δ*bcsA*, cellulose-deficient mutant), AM56 (Δ*pgaA*, PNAG-deficient mutant) were transformed either with the control vector (panel above) or with pYddV (panel below). **B** Surface adhesion on polystyrene microtiter plates by strains carrying either pGEM-T Easy (green bars) or pYddV (red bars). Surface adhesion values are set to 1 for strains transformed with pGEM-T Easy. Actual Adhesion units values were: MG1655(WT)=5.6; AM70(*csgA*)=1.1; LG26(*bcsA*)=5.4; AM56(*pgaA*)=3.8, WT+Dispersin B=4.4. Experiments were repeated three times with very similar results.

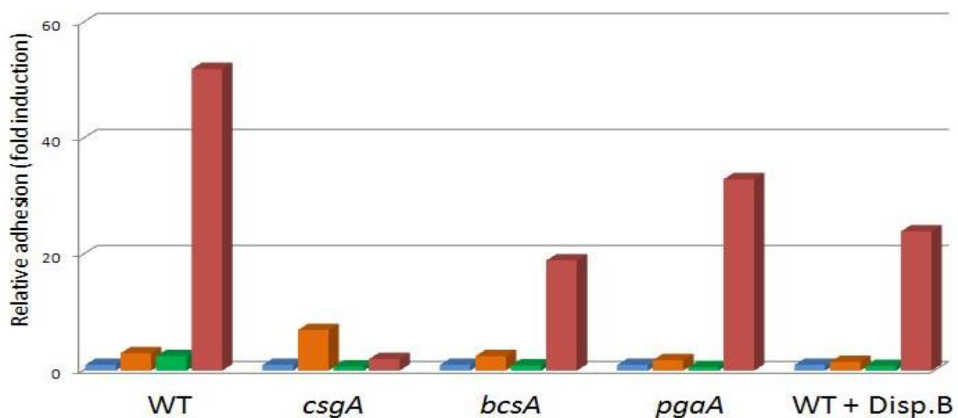


Figure 3.7. Surface adhesion on polystyrene microtiter plates by strains carrying the pGEM-T Easy control vector (blue bars), pAdrA (orange), pYcdT (green), and pYdaM (red). Surface adhesion values are set to 1 for strains transformed with the control vector. Actual values were: MG1655 (WT)=5.6; AM70 (*csgA*)=1.1; LG26 (*bcsA*)=5.4; AM73 (*csgA/bcsA*)=1.2; AM56 (*pgaA*)=3,8, WT+Dispersin B=4.4. Experiments were repeated three times with similar results.

3.2.7 REGULATION OF *pgaABCD* EXPRESSION BY DGCs

As shown in previous chapters, regulation of EPS production by DGCs can take place at different levels, thus we tested the possibility that the YddV protein regulates PNAG production by affecting transcription of the *pgaABCD* operon, encoding the proteins involved in PNAG biosynthesis. We performed quantitative real-time PCR (qRT-PCR) experiments in MG1655 transformed with pYddV and determined the transcript levels of the *pgaA* gene. As shown in Figure 3.8, *pgaA* transcript levels were increased by roughly 10-fold by YddV overexpression. In contrast, overexpression of AdrA and YcdT did not lead to a significant increase in *pgaA* transcript levels. Interestingly, YdaM overexpression also resulted in an increase in *pgaA* transcript levels, albeit lower than that observed for YddV, consistent with YdaM-dependent stimulation of PNAG production (Figure 3.8). To test whether YddV-dependent activation of *pgaABCD* transcription is mediated by its DGC activity, we performed qRT-PCR experiment also with a plasmid carrying a mutant *yddV* allele encoding a protein in which the amino acids in the GGDEF catalytic site were changed to GGAAF ($YddV_{GGAAF}$); as described in sections 2.2.2 and 3.2.2 this mutation results in loss of DGC activity (De *et al.* 2008). Overexpression of the $YddV_{GGAAF}$ protein did not affect *pgaA* transcript levels in real-time PCR experiments (Figure 3.8), suggesting that *pgaABCD* regulation by YddV requires its DGC activity.

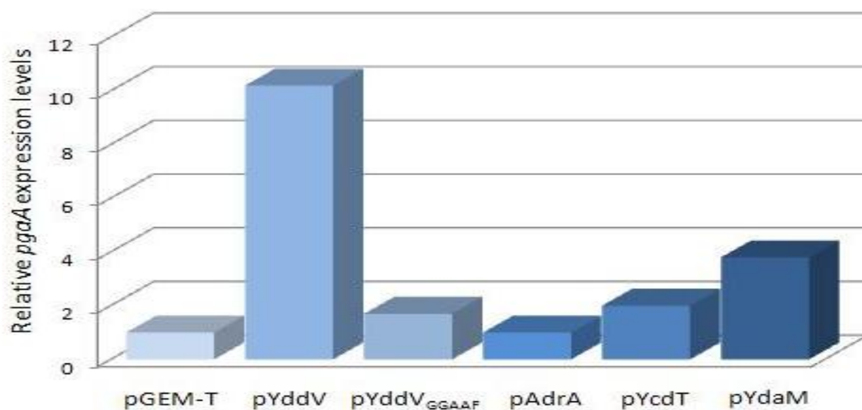


Figure 3.8. Effects of DGC overexpression on *pgaA* transcript levels. The MG1655 strain was transformed either with the pGEM-T Easy vector or with the following plasmids: pYddV, $pYddV_{GGAAF}$, pAdrA, pYcdT, and pYdaM. The pYddV plasmid carries a copy of the wild type *yddV* allele, while $pYddV_{GGAAF}$ carries a mutant *yddV* allele encoding a protein lacking DGC activity. *pgaA* expression values in MG1655 transformed with pGEM-T Easy (corresponding to a Δ_{Ct} relative to 16S rRNA=15.7) was set to 1. The strains were grown overnight in M9 Glu-sup medium at 30°C in the absence of IPTG. Results are the average of three independent experiments performed in duplicate. Standard deviations were always lower than 10%.

3.2.8 YddV POSITIVELY CONTROLS *pgaABCD* EXPRESSION AND PNAG PRODUCTION

To test whether PNAG production is indeed controlled by the *yddV* and *ydaM* genes through *pgaABCD* regulation, we constructed MG1655*yddV* and MG1655*ydaM* mutant derivatives (AM95 and AM89, respectively). In the AM89 strain, the *ydaM* gene is inactivated by the insertion of the EZ-Tn5 <R6Kγori/KAN-2> transposon at nucleotide 654, *i.e.* in the central part of the *ydaM* ORF (1233 bp). AM95 strain carries a *yddV* allele in which the portion of the gene encoding the C-terminal domain 150 amino acids of the YddV protein, which includes the GGDEF domain responsible for DGC activity, has been replaced by a chloramphenicol-resistance cassette (MG1665*yddV*$\Delta_{931\text{-}1383}$::cat; Table 3.4 section 3.4). We measured the effects of the MG1665*yddV*$\Delta_{931\text{-}1383}$::cat mutation on levels of *pgaA* transcript by real-time PCR, which showed that partial deletion of the *yddV* gene resulted in an approximately 3.5-fold reduction in *pgaA* transcript levels in comparison with MG1655 (Figure 3.9). In contrast, no detectable reduction was observed in the MG1655 *ydaM* mutant (AM89), suggesting that the *ydaM* gene is not crucial for *pgaABCD* expression (Figure 3.9).

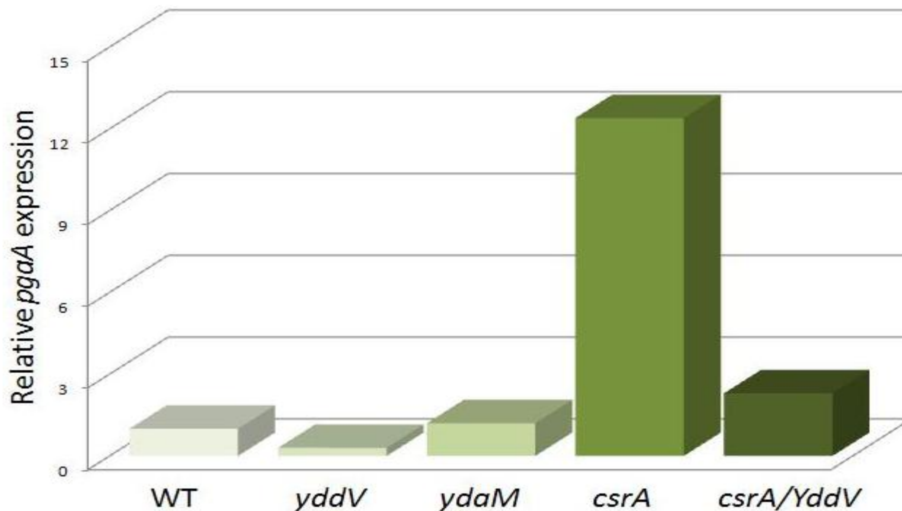


Figure 3.9. Relative expression levels of the *pgaA* gene in strains MG1655 (WT), AM95 (*yddV*), AM89 (*ydaM*), LT24 (*csrA*) and AM98 (*csrA/yddV*), as measured by Real-Time PCR experiments. *pgaA* expression values in MG1655 (corresponding to a ΔC_t relative to 16S rRNA=15.7) was set to 1. Data are the average of three independent experiments, each performed in triplicate. Standard deviations were calculated on the average value of each independent experiment and they were always lower than 5%.

The *pgaABCD* operon is regulated at the transcription initiation level by the NhaR protein, which responds to Na^+ ions (Goller *et al.* 2006). However, the main mechanism of *pgaABCD* regulation takes place at the post-transcriptional level, via negative control by the RNA-binding protein CsrA (Wang *et al.* 2004a, Wang *et al.* 2005; Cerca and Jefferson 2008); CsrA negatively controls *pgaABCD* expression through binding to a 234 nt untranslated region (UTR) in its mRNA, thus blocking its translation and stimulating its degradation (Wang *et al.* 2005). To test whether the YddV protein regulates *pgaABCD* expression by modulating CsrA activity, we constructed AM98, an MG1655*csrA/yddV* double mutant (Table 3.4 section 3.4); the *csrA* mutant allele carried by this strain produces a truncated CsrA protein impaired in its RNA-binding ability, and thus unable to repress *pgaABCD* translation (Mercante *et al.* 2006). As expected, *pgaA* transcript levels were increased by more than 12-fold in the *csrA* mutant strain LT24; the MG1665*yddV*$\Delta_{931-1383}$::cat mutation resulted in a 6-fold reduction in *pgaA* transcript levels in the MG1655*csrA* background (Figure 3.10), indicating that the *yddV* gene positively controls levels of *pgaABCD* transcripts even in a mutant *csrA* background. Thus, YddV does not seem to regulate *pgaABCD* expression by modulating CsrA activity. Since c-di-GMP has been shown to act as a riboswitch, and to be able to increase the chemical and functional half-life of mRNA carrying c-di-GMP-responding elements (Sudarsan *et al.* 2008), we tested the possibility that the *yddV* gene affects *pgaABCD* mRNA stability via its DGC activity. mRNA decay kinetics experiments showed that the *pgaA* transcript has a half-life of ~1.5 min in the MG1655 strain; *yddV* inactivation did not affect *pgaABCD* mRNA stability in the MG1655 background (data not shown), suggesting that *yddV*-dependent *pgaABCD* regulation is not mediated by mRNA stabilization. We investigated the effects of partial deletion of the *yddV* gene on PNAG production by surface adhesion experiments. Surface adhesion to polystyrene microtiter plates is strongly stimulated by inactivation of the *csrA* gene, consistent with higher *pgaABCD* expression in this strain (see Figure 3.9); disruption of the *pgaA* gene, involved in PNAG biosynthesis, counteracts the effects of the *csrA* mutation (Figure 3.10A), indicating that the increased biofilm formation in the *csrA* derivative of MG1655 depends solely on PNAG production. Partial deletion of the *yddV* gene abolished surface adhesion in LT24 (MG1655*csrA* ; Figure 3.10A), consistent with reduced *pgaABCD* expression in AM98 (MG1655*csrA/yddV* mutant; Figure 3.9). Mutations in either the *pgaA* or the *yddV* gene resulted in a 2.5-fold reduction in surface adhesion in the MG1655 background, in agreement with previous observations (Wang *et al.* 2004; see also section 3.2.2). To further confirm that the effects

of *yddV* inactivation on surface adhesion in the MG1655*csrA*/*yddV* background are indeed due to reduced PNAG production, we transformed the AM98 strain with either pYddV, carrying a wild-type copy of the *yddV* gene, or $pYddV_{GGAAF}$, expressing a $YddV_{GGAAF}$ protein lacking DGC activity. Expression of genes cloned into pGEM-T Easy occurs at lower levels in strains carrying a *csrA* mutation, possibly due to reduced plasmid copy number in the *csrA* mutant strain (data not shown); thus, in the absence of IPTG induction, no plasmid was able to restore the ability to form biofilm to AM98 (Figure 3.10B). In contrast, upon IPTG induction, production of YddV, but not of the mutant $YddV_{GGAAF}$ protein lacking DGC activity, clearly stimulated surface adhesion. Treatment with the PNAG-degrading enzyme Dispersin B led to complete loss of biofilm stimulation by the YddV protein (Figure 3.10B), strongly suggesting that the YddV-dependent increase in biofilm formation depends on PNAG production.

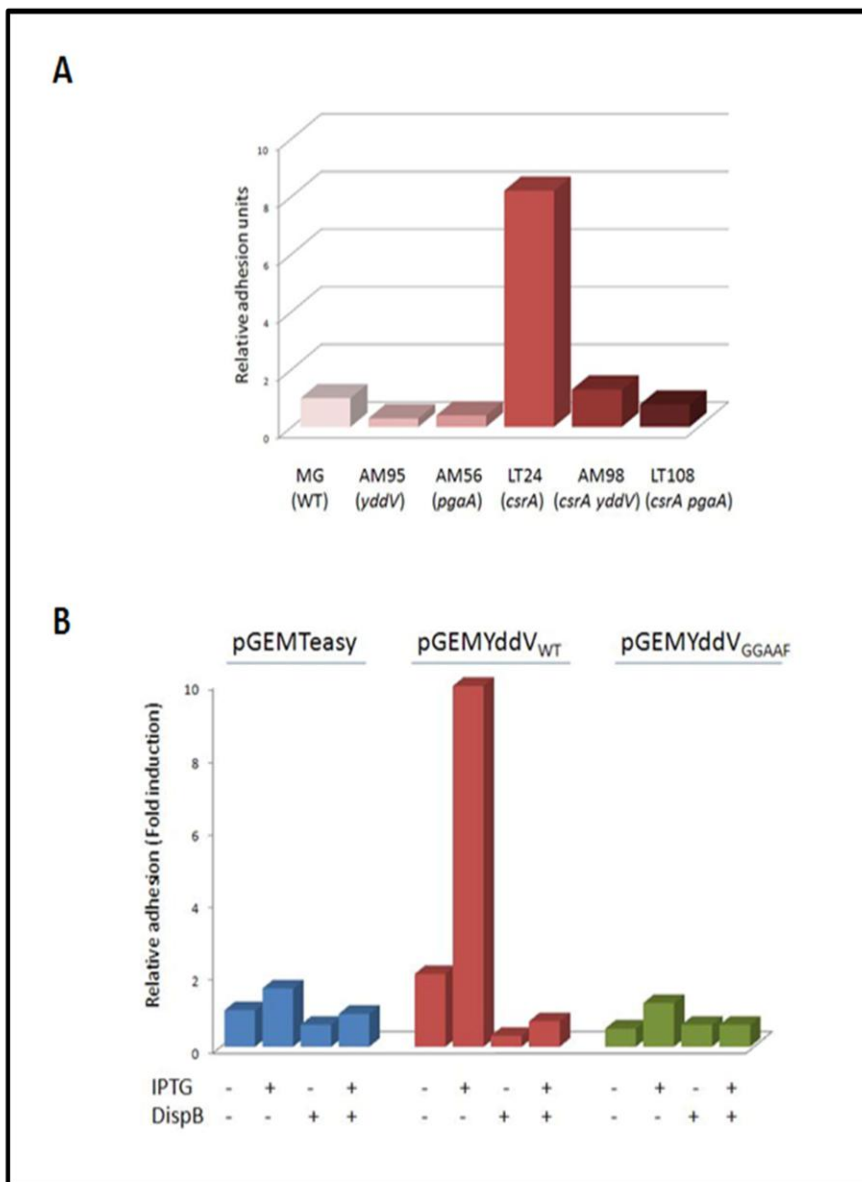


Figure 3.10. **A** Surface adhesion on polystyrene microtiter plates of strains MG1655 (WT), AM95 (*yddV*), AM56 (*pgaA*), LT24 (*csrA*), AM98 (*csrA/yddV*) and LT108 (*csrA/pgaA*). Surface adhesion value for MG1655 (4.9 in this set of experiments) was set to 1. Results are the average of three independent experiments and standard deviations were always lower than 10%. **B** Surface adhesion on polystyrene microtiter plates of strain AM98 (*csrA/yddV*) transformed either with pGEM-T Easy (control vector) or with plasmids carrying *yddV* alleles. The pYddV plasmid carries a copy of the wild type *yddV* allele, while pYddV$_{GGAAF}$ carries a mutant *yddV* allele encoding a protein lacking DGC activity. For full expression, IPTG was added to growth medium at 0.5 mM. When present, Dispersin B was added to the growth medium at a final concentration of 20 µg/ml. Data are the average of two independent experiments with very similar results.

3.3 DISCUSSION

Biosynthesis of the c-di-GMP signal molecule by diguanylate cyclases (DGCs) plays a crucial role in bacterial cell processes such as cell division, motility, biofilm formation, and production of virulence factors (Jenal and Malone 2006; Hengge *et al*. 2009). In Enterobacteria, most DGCs stimulate the transition from motile to sessile cell, repressing flagellar synthesis and cell motility while promoting production of adhesion factors (Mendez-Ortiz *et al.* 2006; Pesavento *et al.* 2008). In this report, we have shown that the DGC-encoding *yddV* gene regulates the expression of genes involved in the production of at least two biofilm determinants: curli and PNAG. Interestingly, beside YddV, at least five proteins involved in c-di-GMP biosynthesis and turnover affect regulation of curli-encoding genes (Weber *et al.* 2006, Sommerfeldt *et al.* 2009; Figure 3.11), thus underlining the tight connection of c-di-GMP signaling system with the production of this important adhesion factor. Indeed, curli (also called thin aggregative fimbriae in *Salmonella*) are probably the major biofilm determinant at low temperatures (≤ 30°C), *i.e.* when Enterobacteria find themselves outside a warm-blooded host. Curli are co-produced with cellulose and, at least in *Salmonella*, with other polysaccharidic components of the outer membrane protein, such as the LPS O-antigen (Gibson *et al.* 2006). In addition to promoting surface adhesion, the curli-polysaccharide matrix allows cell survival to environmental stresses such as desiccation (Gibson *et al.* 2006; Gualdi *et al.* 2008). The *yddV* gene stimulates curli production through control of *csgBAC* expression (Table 3.1), *i.e.*, controlling a regulation step downstream of *csgDEFG* expression.

In addition to curli activation, the *yddV* gene positively regulates the *pgaABCD* operon, encoding PNAG-biosynthetic genes (Figures 3.5, 3.6 and 3.8). PNAG (poly-β-1,6-*N*-acetylglucosamine) is an EPS produced by various bacterial species, including *Y. pestis* and *Staphylococcus* species, where it is also called PIA (polysaccharide intracellular adhesin) and represents a major virulence factor (McKenney *et al.* 1998). Interestingly, PNAG production is activated through c-di-GMP biosynthesis by the HmsT protein in *Y. pestis* (Jones *et al.* 1999, Kirillina *et al.* 2004). In the MG1655 laboratory strain of *E. coli*, the *pgaABCD* operon is only expressed at very low levels (Wang *et al.* 2004a), due to rapid degradation of *pgaABCD* transcript mediated by the CsrA translational repressor (Wang *et al.* 2005). Our results show that a functional *yddV* gene stimulates transcription of *pgaABCD* operon (Figure 3.6). Thus, PNAG production, in addition to CsrA-dependent regulation at translation level, is controlled by c-di-GMP both

at transcription level, via YddV, and at protein stability level, through the effect of the YdeH/YjcC system (Bohem *et al.* 2009; Figure 3.11). Our results suggest that YddV might play a central role in promoting adhesion factors production and biofilm formation in *E. coli* (Figure 3.11). It is likely that YddV works in concert with (a) yet unidentified transcription factor(s) able to bind c-di-GMP.

Figure 3.11 Image adapted from Figure 1.6. Model summarizing the role of YddV (light green ellipse) in the complex network that leads to adhesion factors production in *E. coli*. YddV regulates both curli and PNAG production as indicated by green arrows. Green ellipses indicated DGCs proteins, PDEs are shown as red rectangles.

3.4 MATERIALS AND METHODS

3.4.1 BACTERIAL STRAINS AND GROWTH CONDITIONS

The bacterial strains used in this work are listed in Table 3.4. Bacteria were grown in M9 Glu-sup medium (Brombacher *et al.* 2006; see also section 2.4.1 page 56). For growth under anoxic conditions, liquid cultures were grown with no shaking in 12-mL glass tubes filled to the top; these conditions are sufficient for the full induction of genes responding to anaerobiosis (Landini *et al.* 1994). Antibiotics were used at the following concentrations: ampicillin, 100 μg mL^{-1}; chloramphenicol, 50 μg mL^{-1}; tetracycline, 25 μg mL^{-1}; and kanamycin, 50 μg mL^{-1}. For growth on Congo Red-supplemented or Calcofluor-supplemented agar media, bacteria were inoculated in M9 Glu-sup medium in a microtiter plate, and the cultures were spotted, using a replicator, on CR medium (1% Casamino acids, 0.15% yeast extract, 0.005% $MgSO_4$, 0.0005% $MnCl_2$, 2% agar) to which 0.004% CR and 0.002% Coomassie blue [for Congo Red (CR) medium] or 0.005% Calcofluor [for Calcofluor (CF) medium] were added after autoclaving. Bacteria were grown for 18– 20 h at 30°C; staining was better detected after 24-48 h of additional incubation at 4°C

3.4.2 PLASMID CONSTRUCTION

The plasmids used in this work are listed in Table 3.4. For overexpression of genes encoding DGCs or PDEs, genes of interest were amplified by PCR and the corresponding products cloned into the pGEM-T Easy vector (http://www.promega.com/tbs/tm042/tm042.pdf). Correct orientation of the inserts (*i.e.* under the control of the Plac promoter) was verified by PCR using the primers listed in Table 3.5 For DGC/PDE-overproduction studies, strains carrying pGEM-T Easy derivatives were grown at 30 °C in M9 Glu-sup medium in the absence of IPTG induction of the Plac promoter. The pGEM-$YddV_{GGAAF}$ and pGEM-Dos_{AAA} plasmids were obtained by three-step PCR mutagenesis (Li and Shapiro 1993) using the primers listed in Table 3.5. All constructs were verified by sequencing.

3.4.3 GENE EXPRESSION STUDIES

Quantitative real-time PCR (qRT-PCR) for the determination of the relative expression levels was performed on cultures grown at 30 °C in M9 Glu-sup medium. Samples were taken in the early ($OD_{600\ nm}$=0.25) and late (OD_{600} nm=0.7) exponential phase and in

stationary phase ($OD_{600\ nm}$ 2.5) for cultures grown aerobically, and in stationary phase (OD600 nm 1.6) for cultures grown under anoxic conditions. Primers for real-time PCR are listed in Table 3.5. RNA extraction, reverse transcription and cDNA amplification steps were performed as described (Gualdi *et al.* 2007), using 16S RNA as the reference gene. mRNA stability was measured by real-time PCR experiments in the presence of rifampicin, as described by Wang *et al.* (2005).

Table 3.4 Bacterial strain and plasmids used in this study.

	Relevant genotype or characteristics	**Reference or source**
***E. coli* strains**		
MG1655	Standard reference strain F^-, λ^-, rph-1	Blattner *et al.* 1997
EB1.3	MG1655 *rpoS::tet*	Prigent-Combaret *et al.* 2001
PHL856	MG1655 *csgA-uidA::Kan*	Gualdi *et al.* 2008
LG26	MG1655Δ*bcsA::kan*	Gualdi *et al.* 2008
AM56	MG1655Δ*pgaA::cat*	This thesis
AM70	MG1655 *ΔcsgA::cat*	This thesis
AM73	MG1655 *ΔcsgA::cat, ΔbcsABZC::kan*	This thesis
AM75	MG1655*csgD::cat*	This thesis
AM89	MG1655*ydaM::Tn5-kat*	This thesis
AM95	MG1665*yddV*$\Delta_{931\text{-}1383}$::cat	This thesis
AM98	MG1655*csrA*::Kan,*yddV*$\Delta_{931\text{-}1383}$*::cat*	This thesis
AM109	MG1655*dos::tet*$\Delta_{1200\text{-}2400}$	This thesis
LT24	MG1655 *csrA::Tn5-kan* Obtained by bacteriophage P1 transduction from TRMG1655 (Romeo *et al.* 1993)	This thesis
LT108	MG1655*csrA::kan ΔpgaA::cat*	This thesis
Plasmids		
pGEM-T Easy	Control vector allowing direct cloning of PCR products, ampicillin and kanamycin resistance	Promega
pGEM-YddV$_{WT}$ (pYddV)	*yddV* gene cloned as PCR product into pGEM-T Easy vector	This thesis
pGEM-YddV$_{GGAAF}$	*yddV* allele carrying mutation resulting in GGDEF→GGAAF change in YddV DCG catalytic site	This thesis
pGEM-Dos$_{WT}$	*dos* gene cloned as PCR product into pGEM-T Easy vector	This thesis
pGEM-Dos$_{GGAAF}$	*dos* allele carrying mutation resulting in EAL→AAA change in Dos PDE protein catalytic site	This thesis
pGEM-AdrA (pAdrA)	*adrA* gene cloned as PCR product into pGEM-T Easy vector	This thesis
pGEM-YcdT (pYcdT)	*ycdT* gene cloned as PCR product into pGEM-T Easy vector	This thesis
pGEM-YdaM (pYdaM)	*ydaM* gene cloned as PCR product into pGEM-T Easy vector	This thesis

Table 3.5 Primers used in this study.

Primers	Sequence	Utilization
yddV_for	5'-CCAGCCTTATAAGGGTGTG-3'	*yddV* cloning and mutant verificatio
yddV_rev	5'-TTACCTCTGCATCCTGGC-3'	*yddV* cloning
$yddV_{GGAAF}$_for	5'-TACGGGGGCG**C**TG**C**ATTTATCATT-3'	Construction of pGEM-$YddV_{GGAAF}$
$yddV_{GGAAF}$_rev	5'-AATGATAAAT**G**CA**G**CGCCCCCGTA-3'	Construction of pGEM-$YddV_{GGAAF}$
dos_for	5'-AATCATGAAGCTAACCGATGCG-3'	*dos* cloning
dos_rev	5'-TTGTCAGATTTTCAGCGGTAACAC-3'	*dos* cloning
dos_{AAA}_for	5'-ACGGCATCG**C**AGCC**GC**TGCTCGCT-3'	Construction of pGEM-Dos_{AAA}
dos_{AAA}_rev	5'-AGCGAGCA**GC**GGCT**G**CGATGCCGT-3'	Construction of pGEM-Dos_{AAA}
adrA_for	5'-GCTCCGTCTCTATAATTTGGG-3'	*adrA* cloning
adrA_rev	5'-ATCCTGATGACTTTCGCCGG-3'	*adrA* cloning
ydaM_for	5'-GCGATCGGATAGCAACAA-3'	*ydaM* cloning and mutant screenin
ydaM_rev	5'-GAAGTCGTTGATCTCGAC-3'	*ydaM* cloning and mutant screenin
EZ-Tn5_for	5'-CCTCTTTCTCCGCACCCGAC-3'	*ydaM* mutant screenir
ycdT_for	5'-GGGATCTACAACCTACAG-3'	*ycdT* cloning
ycdT_rev	5'-CATATTACGTGGGTAGGATC-3'	*ycdT* cloning
yddV_cat_for	5'-GGATGTACTGACGAAATTACTTAACCG CCGTTTCCTACCGTACCTGTGACGGAAGATCAC-3'	*yddV* inactivation
yddV_cat_rev	5'-CATCGGTTAGCTTCATGATTACCTCTGC ATCCTGGCGCATGGGCACCAATAACTGCCTTA-3'	*yddV* inactivation
dos_tet_for	5'-CCTGCACAATTACCTCGATGACCTGGTCGACAA AGCCGTCCTAGACATCATTAATTCCTA-3'	*dos* inactivation
dos_tet_rev	5'-GTTAAATGAAAACCCGCGAGTGCGGGCGAGAG GAATTTGGAAGCTAAATCTTCTTTATCG-3'	*dos* inactivation
csgD_cam_for	5'-CTGTCAGGTGTGCGATCAATAAAAAAAGCGG GGTTTCATCTACCTGTGACGGAAGATCAC-3'	*csgD* inactivation
csgD_cam_rev	5'-AATGAATCAGGTAGCTGGCAAGCTTTTGCGTAA AGTAGCAGGGCACCAATAACTGCCTTA-3'	*csgD* inactivation
csgA_cat_for	5'-TTTCCATTCGACTTTTAAATCAATCCGATGG GGGTTTTACTACCTGTGACGGAAGATCAC-3'	*csgA* inactivation
csgA_cat_rev	5'-AACAGGGCTTGCGCCCTGTTTCTGTAATACA AATGATGTAGGGCACCAATAACTGCCTTA-3'	*csgA* inactivation
pgaA-cat_for	5'-ATACAGAGAGAGATTTTGGCAATACAT GGAGTAATACAGGTACCTGTGACGGAAGATCAC-3'	*pgaA* inactivation
pgaA-cat_rev	5'-ATCAGGAGATATTTATTTCCATTACGTA ACATATTTATCCGGGCACCAATAACTGCCTTA-3	*pgaA* inactivation
csgD_rev	5'-GCCATGACGAAAGGACTACACCG-3'	Mutant verification
cat_rev	5'-GGGCACCAATAACTGCCTTA-3'	Mutant verification
tet_rev	5'-GAAGCTAAATCTTCTTTATC-3'	Mutant verification
csgA_for	5'-ACAGTCGCAAATGGCTATTC-3'	Mutant verification
pgaA_for	5'-TGGACACTCTGCTCATCATTT-3'	Mutant verification
16S_for	5'-TGTCGTCAGCTCGTGTCGTGA-3'	qRT-PCR
16S_rev	5'-ATCCCCACCTTCCTCCGGT-3'	qRT-PCR
csgB_RT_for	5'-CATAATTGGTCAAGCTGGGACTAA-3'	qRT-PCR
csgB_RT_rev	3'-GCAACAACCGCCAAAAGTTT-3'	qRT-PCR
csgD_RT_for	5'-CCCGTACCGCGACATTG-3'	qRT-PCR
csgD_RT_rev	5'-ACGTTCTTGATCCTCCATGGA-3'	qRT-PCR
dos_RT_for	5'-CAGAGAAGCTCTGGGGATACA-3'	qRT-PCR and mutant verification
dos_RT_rev	5'-TTTTTCTCCAGCTGCAGCTCC-3'	qRT-PCR
pgaA_RT_for	5'-CCGCTACCGTCATCAGCAATT-3'	qRT-PCR
pgaA_RT_rev	5'-AGCGCCTTTTGCCACAGTGT-3'	qRT-PCR

3.4.4 BIOFILM FORMATION ASSAYS

Biofilm formation in microtiter plates was determined as described in section 2.4.2 page 58. The sensitivity of biofilms to treatment with the PNAG-degrading enzyme Dispersin B (Kaplan *et al.* 2004; purchased from Kane Biotech) was determined by adding 20 μg mL^{-1} enzyme to the growth medium.

3.4.5 OTHER METHODS

E. coli MG1655 mutant derivatives were constructed either using the λ Red technique (Datsenko and Wanner, 2000) or by bacteriophage P1 transduction (Miller 1972), except for the AM89 strain (MG1655 *ydaM*::Tn5-kan), which was obtained in a transposon mutagenesis screening for adhesion-deficient MG1655 mutants using the EZ-Tn5 <R6Kγori/KAN-2> Transposon (Epicentre; Landini unpublished data). Primers used for gene inactivation and for confirmation of target gene disruption by PCR are listed in Table 3.5. Bacterial cell motility was evaluated as described by Pesavento *et al.* (2008). Determination of intracellular c-di-GMP concentration was performed as described in section 2.4.4 page 59.

CHAPTER IV

pnp INACTIVATION RESULTS IN POLY-*N*-ACETYLGLUCOSAMINE OVERPRODUCTION IN *E. coli* C

4.1 INTRODUCTION

As described in previous chapters the second messenger cyclic-di-GMP (c-di-GMP) can trigger production of adhesion factors and promote biofilm formation (Hengge 2009). The ability of c-di-GMP to affect cellular behavior relies on the diversity of c-di-GMP receptors, indeed, c-di-GMP can interact with several targets, such as transcriptional regulators FleQ and Clp (Hickman and Harwood 2008; Chin *et al.* 2010), or PilZ domain of BcsA protein, determining an allosteric activation of cellulose production (Amikam and Galperin 2006); in additions, c-di-GMP interacts with RNA domain (riboswitch) affecting RNA stability and translation (Sudarsan *et al.* 2008). In general these receptors monitor the c-di-GMP level in the cell and translate it into a specific behavioral response (Sondermann *et al.* 2012). Recently Tuckerman *et al.* (2011) showed direct interaction between c-di-GMP and the mRNA processing enzyme polynucleotide phosphorylase (PNPase) suggesting that PNPase could act in combination with c-di-GMP in gene regulation at post transcriptional level.

PNPase is an evolutionarily conserved enzyme affecting gene expression in bacteria, plants, and mammals (Sarkar and Fisher 2006). In *E. coli*, PNPase acts as a 3' exoribonuclease which cleaves phosphodiester bonds using phosphate as cofactor (phosphorolysis). Nevertheless, it has been reported that PNPase can add 3'-polynucleotide extensions to some mRNAs or to fragments derived from mRNA decay (Mohanty and Kushner 2000; Mohanty and Kushner 2006). Although intriguing, the physiological relevance of the synthetic reaction remains to be elucidated (Carpousis *et al.* 2007).

Despite evidence of direct c-di-GMP-PNPase interaction, the exact mechanism of action of c-di-GMP-PNPase complex is still not known. In this chapter I have studied the possible connections between PNPase and c-di-GMP-dependent gene regulation and biofilm formation. We observed that the lack of a functional *pnp* gene results in cellular aggregation due to poly-*N*-acetylglucosamine (PNAG) increased production. However, although PNPase regulates expression of PNAG-related genes this effect does not seem to depend on c-di-GMP.

4.2 RESULTS

4.2.1 AGGRGATION AND BIOFILM FORMATION IN *E. coli* C *pnp*⁻ STRAIN (C-5691)

The strains described in this chapter were the wild type *E.coli* C, C-1a (Sasaki and Bertani 1965; Table 4.5) and its *pnp* deletion mutant derivative C-5691 (from Gianni Dehò's laboratory, University of Milan; Table 4.5). Overnight cultures showed that C-5691 has a strong aggregative phenotype, indicative of adhesion factors production (Figure 4.1A). Cell aggregation occurs at the transition between exponential and stationary phase and results in cell clumping and reduction in OD (data not shown). The aggregative phenotype was stronger when cells were grown in M9 Glucose supplemented medium (M9 Glu-sup; see section 4.4.1) at 37°C (Figure 4.1A). To confirm these observations we tested both wild type strain C-1a and *pnp* mutant C-5691 in standard crystal violet assay in M9 Glu-sup both at 30°C and 37°C. The *pnp* mutation strongly stimulates biofilm formation in *E. coli* C (Figure 4.1B and C). In particular results at 37°C show a very good correlation with aggregation assays, suggesting that in *E. coli* C the lack of *pnp* gene might induce production of adhesion factors able to promote efficient aggregation and biofilm formation.

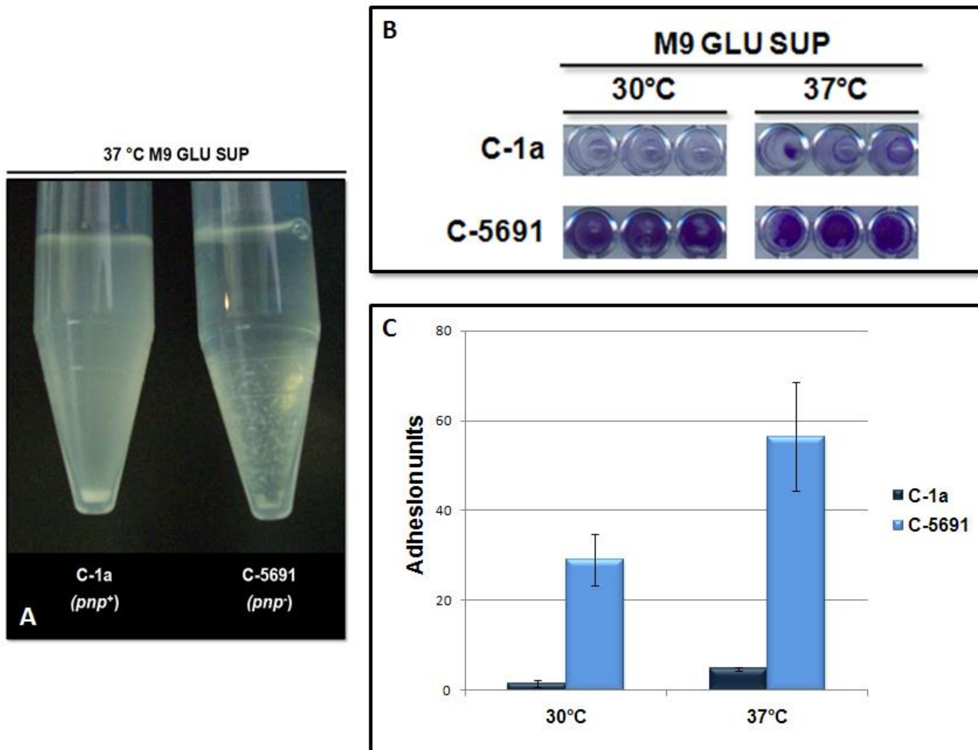
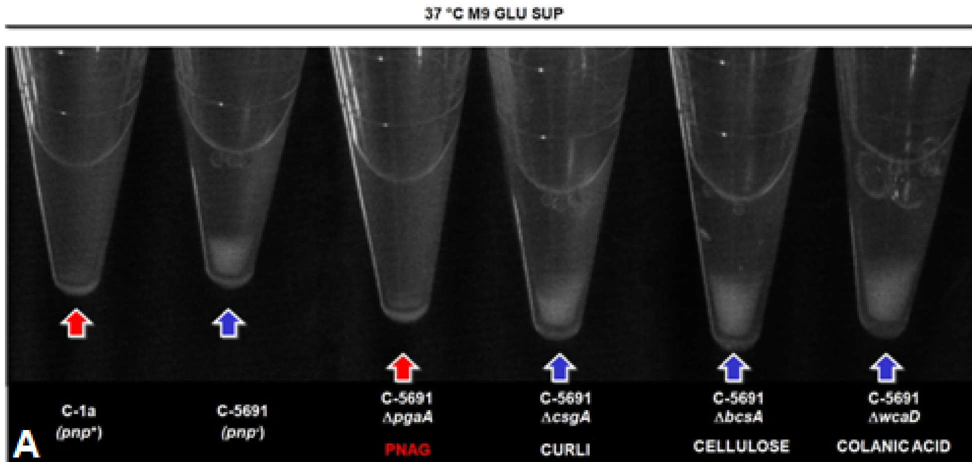


Figure 4.1 A Effects of lack of functional *pnp* gene on aggregation in *E.coli C* at 37°C in M9 Glu-sup medium. **B** Biofilm formation, measured with the crystal violet assay, in C-1a (*pnp*$^{+}$) and C-5691 (*pnp*$^{-}$) at 30°C and 37°C in M9 Glu-sup medium. Experiments are performed in triplicate. **C** Semi-quantitative evaluation of biofilm in crystal violet assays gave adhesion values of 1,57 and 4,78 for C-1a grown at 30°C and 37°C respectively (dark blue bars) and 29,05 and 56,57 for C-5691 grown at 30°C and 37°C respectively (light blue bars). Results are the average of three independent experiments, error bars are shown.

4.2.2 THE AGGREGATIVE PHENOTYPE IN *pnp*$^{-}$ MUTANT DEPENDS ON POLY-*N*-ACETYLGLUCOSAMINE (PNAG) PRODUCTION

In order to identify which adhesion factor promotes aggregation and biofilm formation in C-5691, we created a set of isogenic C-5691 (*pnp*$^{-}$) derivatives deficient in the production of known *E. coli* biofilm determinants, namely: curli (C-5691Δ*csgA*), cellulose (C-5691Δ*bcsA*), colanic acid (C-5691Δ*wcaD*) and PNAG (5691Δ*pgaA*), and we tested their adhesion abilities in M9 Glu-sup at 37°C.



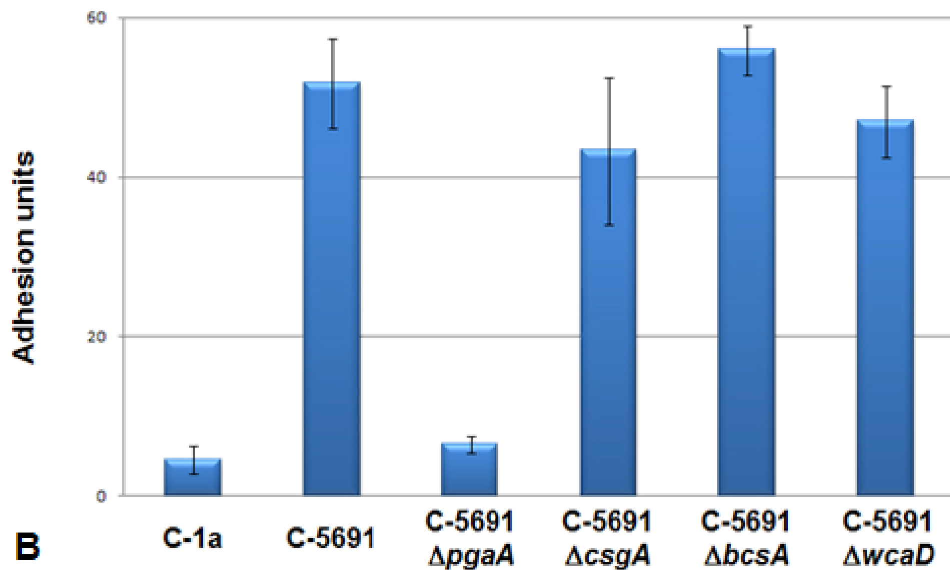


Figure 4.2 A Aggregation proprieties of C-1a, C-5691 and C-5691 isogenic mutants deficient in the production of PNAG (5691Δ*pgaA*), curli (C-5691Δ*csgA*), cellulose (C-5691Δ*bcsA*), colanic acid (C-5691Δ*wcaD*). The blue and red arrows indicate respectively aggregative and non-aggregative phenotypes. All strains were grown in M9 Glu-sup at 37°C. **B** Semi-quantitative evaluation of C-1a, C-5691 and C-5691 derivatives biofilm formation with crystal violet assays. Adhesion values were 4,53 (C-1a), 51,79 (C-5691), 6,49 (C-5691Δ*pgaA*), 43,28 (C-5691Δ*csgA*), 55,94 (C-5691Δ*bcsA*) and 46,99 (C-5691Δ*wcaD*). Results are the average of three independent experiments, error bars are shown.

As shown in the Figure 4.2 only inactivation of PNAG biosynthetic gene *pgaA* totally suppressed cell aggregation (Figure 4.2A) and adhesion to microtiter plate (Figure 4.2B) in the C-5691 background. As described in 4.2.1 we decided to test biofilm formation of C-1a, C-5691 and C-5691 isogenic derivatives.

In contrast, inactivation of genes responsible for the production of other adhesion factors showed little or no effects (Figure 4.2).

These results strongly suggest that cell aggregation and biofilm formation in C-5691 mutant are exclusively mediated by PNAG production. Since PNPase is involved in RNA processing (Carpousis 2007), we hypothesized that lack of functional PNPase would affect PNAG production via regulation of transcript levels of the PNAG biosynthetic operon *pgaABCD*, thus leading to increased PNAG production. In order to test this hypothesis we performed quantitative real-time PCR (qRT-PCR) experiments to determine relative amounts of the *pgaA* transcript both C-1a (*pnp*$^{+}$) and C-5691 (*pnp*$^{-}$) strains: results clearly indicate that in C-5691 *pgaA* gene expression is significantly higher than in C-1a (Figure 4.3). Since many adhesion factors are regulated in a temperature-dependent manner (*e.g.* curli; Olsén *et al.* 1993) we tested *pgaA* gene expression both at 30°C and 37°C: expression was increased in *pnp*$^{-}$ background with a slightly enhanced effect at 37°C (12-fold *vs.* 6-fold induction). (Figure 4.4) suggesting that growth temperature does not play a major role in *pga* locus regulation

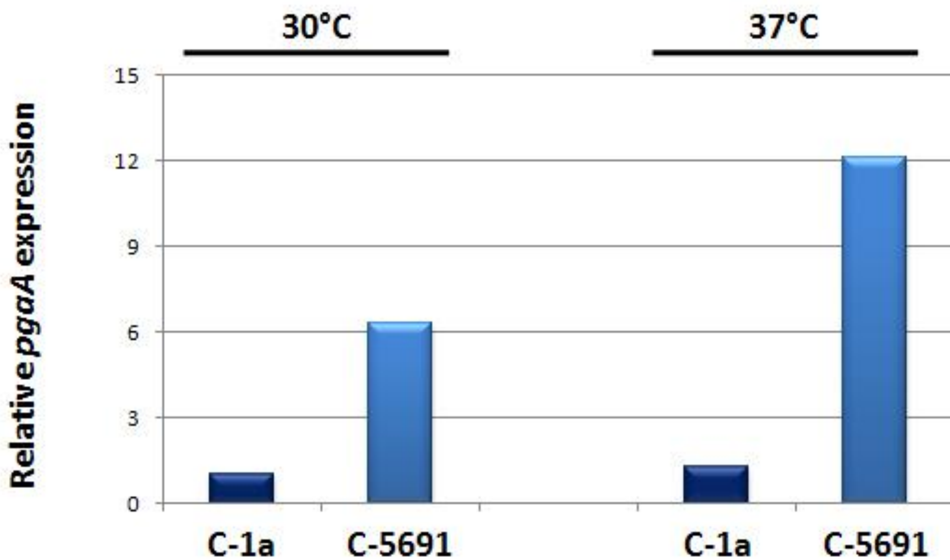


Figure 4.3 Relative expression levels of the *pgaA* gene in strains C-1a and C-5691 grown in M9 Glu-sup medium at 30°C and 37°C, as measured by qRT-PCR experiments. *pgaA* expression values in C-1a grown at 30°C (corresponding to a Δ*Ct* relative to 16S rRNA=10.7) was set to 1. Results are the average of six independent experiments, with very similar results.

4.2.3 5'-UTR OF *pgaABCD* TRANSCRIPT IS NECESSARY FOR PNPase-DEPENDENT REGULATION

The *pgaABCD* 5'-UTR region is the target for regulation by CsrA, an RNA-binding protein which represses translation of the *pgaABCD* transcript and mediates the main machinery of *pgaABCD* regulation (Wang *et al.* 2005; Suzuki *et al.* 2006). In turn, CsrA can be sequestered by the small RNAs CsrB and CsrC, leading to translational de-repression (Suzuki *et al.* 2006). Since PNPase is involved in RNA processing, we expected that it could act either directly on the 5'-UTR or affecting the processing of small RNAs CsrB and CsrC. Thus, in order to test whether PNPase directly regulates *pgaABCD* targeting the operon 5'-UTR, we constructed plasmids in which the *luxAB* reporter was placed under the control either of the *pgaABCD* regulatory elements (promoter and 5'-UTR) or of the promoter region alone (ΔUTR construct; Figure 4.4).

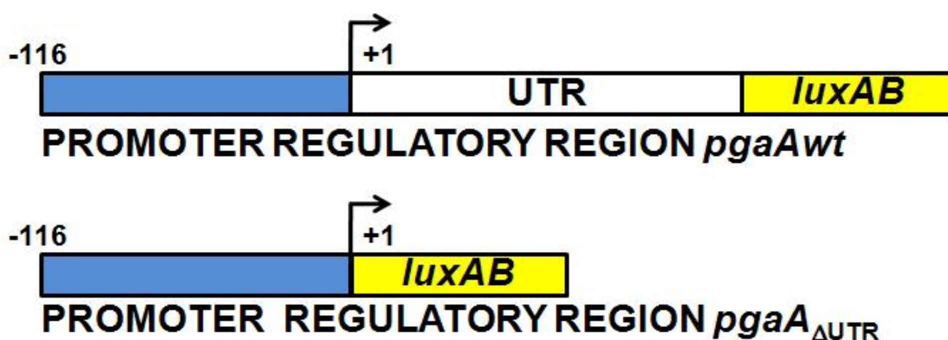


Figure 4.4 Scheme of the promoter regulatory region of *pgaAwt* and $pgaA_{\Delta UTR}$. The figure is not in scale.

Results of luciferase assays show that in a *pnp*$^-$ mutant *pgaABCD* expression is only increased when *luxAB* is placed under the control of the entire *pgaABCD* promoter region (5'-UTR and promoter; Table 4.1). In contrast, when we performed experiments with plasmid carrying a complete deletion of 5'-UTR we observed both in C-1a and C-5691 a strong stimulation of *pgaABCD* expression and PNPase-dependent downregulation was totally abolished (Table 4.1). Thus our results showed that the presence of the 5'-UTR is necessary for PNPase-dependent regulation and suggesting that a *pnp* mutation relieves (only partially) the negative effect by UTR.

Table 4.1 Luciferase assay results (luciferase units) obtained from overnight cultures grown in M9 Glu-sup medium at 37°C. Results are the average of four independent experiments.

	C-1a	C-5691	Fold-induction (C-5691/C-1a)
+ *pgaA* 5'-UTR	38,32 ± 7,8	166,4 ± 11,0	4,3
- *pgaA* 5'-UTR	538,6 ± 51,3	703,4 ± 38,4	1,3

4.2.4 DETERMINATION OF PNAG PRODUCTION

To further confirm that both aggregation and biofilm formation are mediated by increased PNAG production we carried out a dot-blot analysis using a specific anti-PNAG antibody comparing C-5691(*pnp*$^-$) to its parental strain. Since we observed stronger *pnp*-dependent aggregation and *pgaA* gene expression at 37°C in M9 Glu-sup, we measured PNAG from samples grown in these conditions. As expected from the results presented in previous sections, PNAG production was increased in the C-5691 strain (Figure 4.5 first column). As control for PNAG-specificity of the antibody we also tested the effect of *pgaA* mutation, both in C-1a and C-5691 genetic background. Neither strain showed any reactivity with the anti-PNAG antibody (Figure 4.5). Finally we monitored PNAG production in strains carrying mutations affecting production of other adhesion factors. Interestingly, while PNAG production was not affected in the Δ*csgA* and in the Δ*wcaD* strains, it was clearly enhanced in the Δ*bcsA* mutant, unable to produce cellulose (Figure 4.5). This result seems to suggest that cellulose production might impair PNAG biosynthesis. Interestingly, lack of cellulose and stimulation of PNAG production leads to increased cell aggregation (Figure 4.5; Table 4.2), suggesting that cellulose is a negative determinant for cell adhesion in *E. coli* C.

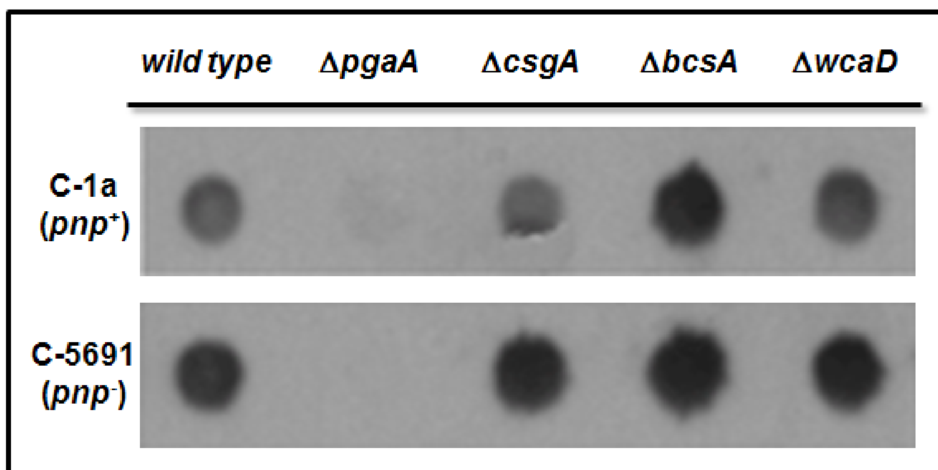


Figure 4.5 PNAG detection by dot-blot analysis. PNAG was detected in each strain we tested, except, for strains carrying a non functional PNAG export machinery (Δ*pgaA* second column) as expected. Results obtained from strains grown overnight in M9 Glu-sup medium at 37°C

Table 4.2 Effects of mutations in adhesion factors on cell aggregations. Results obtained from strains grown overnight in M9 Glu-sup medium at 37°C

	Wild type	Δ*pgaA*	Δ*csgA*	Δ*bcsA*	Δ*wcaD*
C-1a (*pnp*$^+$)	-	-	-	++	-
C-5691(*pnp*$^-$)	+++	-	+++	+++	+++

Aggregation determined by visual inspection as described in Gualdi *et al.*, 2008. Results are obtained from four independent experiments.

4.2.5 EFFECTS OF *pnp* MUTATION ON OUTER MEMBRANE PROTEINS (OMPs) PATTERN

In order to investigate if lack of a functional PNPase and increased PNAG production might have effects on cell surface proteins pattern, we analyzed OMPs in C-1a and C-5691 grown in M9 Glu-sup at 37°C on monodimensional SDS-PAGE. As shown in Figure 4.7 the lack of functional *pnp* gene only resulted in slight modifications in the OMPs pattern, *i.e.* a changes in the relative intensity of bands 1 and 2 and the appearance of a faint band at an apparent molecular weight of 22KDa (3).The band were excises and were identified by MALDI-TOF after in-gel trypsin digestion (Table 4.3).

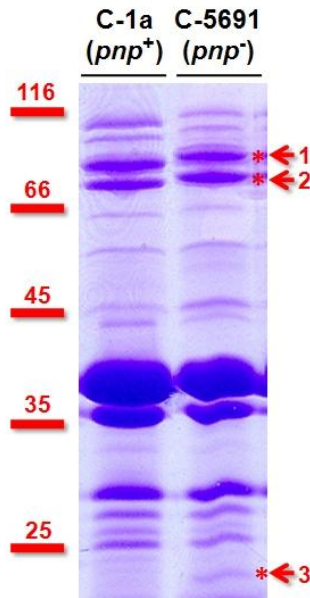


Figure 4.6 SDS-PAGE of fractionated cell extracts. Gel corresponding to outer membrane proteins (OMPs) fraction. Lane 1 C-1a (*pnp*$^+$); lane 2 C-5691 (*pnp*$^-$). The position of molecular mass markers is shown (numbers indicate molecular masses in kilodaltons). Asterisks indicate bands differentially expressed in a *pnp*$^-$ background that were excised and identified by MALDI-TOF (numbered from 1 to 3).

Band analysis by MALDI-TOF allowed detection of several polypeptidic chains within the same band and their relative quantification. Consistent with previous observation, PgaA, the main OMP component of PNAG biosynthetic machinery was only detected in C-5691. In addition, in a *pnp*$^-$ background we found stimulation of other proteins: Fiu, involved in iron uptake in band 2, GlgB, involved in glycogen biosynthesis in band 1 together with PgaA and finally SpeG, a subunit of spermidine acetyltransferase in band 3 (Table 4.3). In contrast in C-5691 the lack of a functional *pnp* gene negatively affected the production of LptD, which is involved in lipopolysaccharide biosynthesis (Table 4.3).

Band N°	Gene product	Predicted molecular mass (kDa)[A]	Function (reference)
1	PgaA *	92,207	Required for PNAG biosynthesis. PgaA exports PNAG across the outer membrane (Wang *et al.* 2005).
1	GlgB *	84,337	1,4-α-glucan branching enzyme (Abad *et al.* 2002).
2	Fiu *	81,969	Putative outer membrane receptor for iron transport It facilitates the uptake of siderophore dihydroxybenzoylserine (Hantke 1990) and serves as the receptor for microcins E492, M, H47 (Patzer *et al.* 2003).
2	LptD†	89,671	The LptD (**l**ipo**p**olysaccharide **t**ransport) protein is an essential outer membrane (OM) protein which, in complex with LptE, functions to assemble lipopolysaccharides at the surface of the OM (Wu *et al.* 2006)
3	SpeG *	21,887	Subunit composition of spermidine acetyltransferase (Limsuwun and Jones 2000).

Table 4.3 Gene characteristics
* Increased expression in C-5691 (*pnp*$^-$); † decreased expression in C-5691 (*pnp*$^-$).
[A] Predicted molecular masses were obtained from the EcoCyc database (http://www.ecocyc.org/).

This results could suggest that PNPase might control expression of the genes *glgB*, *fiu*, *lptD* and *speG*. Thus, through qRT-PCR analysis we tested if *pnp* deletion could affect transcript stability of these genes. As shown in Table 4.4 only *glgB* transcription is slightly stimulated in C-5691 (c. 1,8-fold). In contrast no differences were detected in a *pnp*$^-$ background on other genes expression. Thus last results suggest that the *pnp* mutation might affects production of the proteins Fiu, GlgB, SpeG and LptD only indirectly; it is possible that increased PNAG production in C-5691 rather than the *pnp* mutation itself might lead to reorganizations of the OMPs pattern.

Table 4.4 Relative expression of *glgB*, *fiu*, *lptD* and *speG* genes in C-1a (pnp^+) vs. C-5691 (pnp^-). Results obtained from strains grown overnight in M9 Glu-sup medium at 37°C

glgB		***fiu***		***lptD***		***speG***	
C-1a pnp^+	**C-5691** pnp^-	**C-1a** pnp^+	**C-5691** pnp^-	**C-1a** pnp^+	**C-5691** pnp^-	**C-1a** pnp^+	**C-5691** pnp^-
1*	1,79	1*	0,89	1*	0,87	1*	1,12

*ΔC_t between the gene of interest and the 16S gene was arbitrarily set at 1 for C-1a stationary phase of growth. The actual ΔC_t values were: *glgB*=8,1; *fiu*=8,05; *lptD*=8,8 *speG*=8,45. ΔC_t between the gene of interest and the 16S gene for mutant strain are expressed as relative values. Values are the average of two independent experiments performed in duplicated with very similar results.

4.2.6 EFFECTS OF *dos* INACTIVATION ON *pgaABCD* TRANSCRIPTION

Recently it has been discovered that PNPase co-purifies with the YddV-Dos complex (Tuckerman *et al.* 2011) which encodes for a diguanylate cyclase (DCG) and a phosphodiesterases (PDE) respectively (Méndez-Ortiz *et al*. 2006). Tuckerman and colleagues indicates that specific YddV-mediated c-di-GMP production leads to a PNPase activation (Tuckerman *et al.* 2011). Since Dos counteracts with its PDE activity YddV (Tuckerman *et al.* 2009), in a *dos* mutant we expected an higher YddV DCG-activity and consequently a stimulation of PNPase activity. In other words, a *dos* mutant should display opposite effects than a *pnp* deleted strain. We observed that *pgaABCD* transcription is not affected in a C-1a *dos* mutant (namely AD01; Table 4.5) in which the distal part of the gene that contains EAL domain required for PDE-activity is replaced by the tetracycline (*tet*) gene. Unfortunately I was not able to transduce the *dos* mutation in a pnp^- background; indeed C-5691 is not suitable for transduction and genetic manipulations since it shows lower level of homologous recombination than *wild-type* (Cardenas *et al.* 2011). However our results showed that *dos* mutation does not affect in any way *pgaABCD* transcription in a C-1a context at 37°C (Figure 4.7).

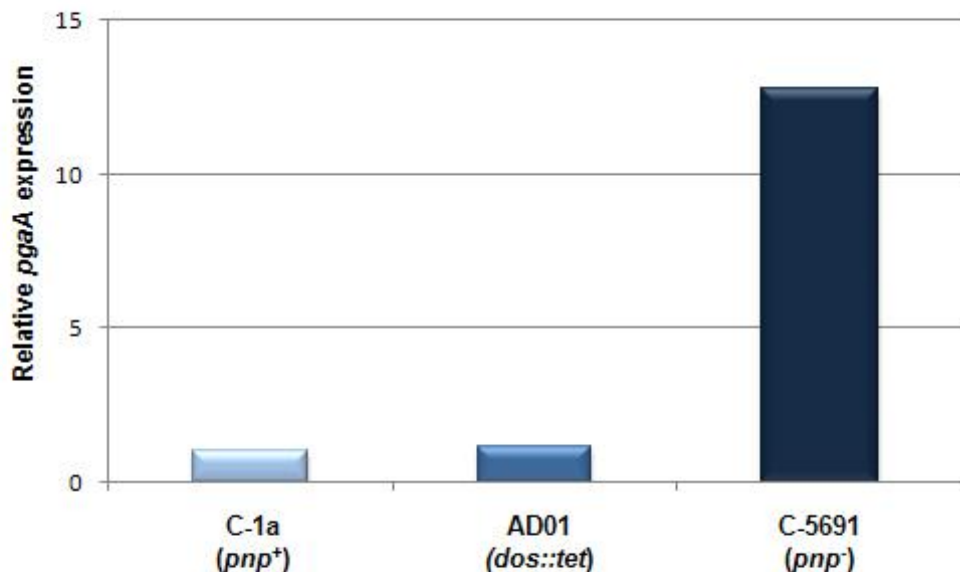


Figure 4.7 Relative expression levels of the *pgaA* gene in strains C-1a, AD01 (C-1a *dos::tet*$\Delta_{1200\text{-}2400}$) and C-5691 grown in M9 Glu-sup medium 37°C, as measured by qRT-PCR experiments. *pgaA* expression values in C-1a grown at 37°C (corresponding to a ΔCt relative to 16S rRNA=9.9) was set to 1. Results are the average of two independent experiments, with very similar results.

These results suggest that PNPase control *pgaABCD* transcription through a mechanism independent of c-di-GMP.

4.3 DISCUSSION

In bacteria biofilm formation is subjected to a complex regulation; a pivotal role in switch between planktonic and biofilm lifestyles is played by the signal molecule c-di-GMP (Sondermann *et al.* 2012). c-di-GMP modulates cellular behaviour interacting either with protein presenting c-di-GMP binding domain, *i.e.* PilZ or RxxD (Amikam and Galperin 2006; Lee *et al.* 2007) or with transcriptional regulator such as FleQ or Clp (Hickman and Harwood 2008; Chin *et al.* 2010); in addition c-di-GMP regulates directly gene transcription acting with mRNA riboswitch (Sudarsan *et al.* 2008). Recently it has been demonstrated that c-di-GMP directly interact with the mRNA processing enzyme PNPase suggesting a c-di-GMP-dependent mRNA processing in cells through direct protein-protein interaction

between YddV and PNPase (Tuckerman *et al.* 2011). Thus we studied the possible connections between PNPase and c-di-GMP-dependent gene regulation in biofilm formation. As described in Chapter III *pgaABCD* operon, required for PNAG export biosynthesis and assembly (Itoh *et al.* 2008), is positively regulated by YddV through its diguanylate cyclase activity. Based on these observations we expected that PNPase could be a positive regulator of *pgaABCD* expression.

However, lack of a functional *pnp* gene results in aggregation due to poly-*N*-acetylglucosamine (PNAG) increased production (Figure 4.5); and stimulates transcription of *pgaABCD* operon (Figure 4.3). The presence of the 5'-UTR of the *pgaABCD* transcript is necessary for PNPase-dependent regulation (Table 4.1). Thus, our results suggest that PNPase control *pgaABCD* transcription through an unknown mechanism in which YddV-mediated c-di-GMP production is not involved.

In addition to PNAG overexpression a *pnp* deletion caused slight modifications in the outer membrane proteins (OMPs) pattern (Figure 4.6) affecting production of GlgB, Fiu, LptD and SpeG. In particular a reduction in LptD protein which is involved in lipopolysaccharide biosynthesis might indicate that cells re-organise the structures of outer membrane in response to PNAG overproduction regulating in a negative way other adhesion determinants *e.g.* lipopolysaccharide and other EPS. Likewise we observed that cellulose negatively affected PNAG production both in wild type and mutant strains (Figure 4.5) suggesting that different EPS might be naturally regulated to keep their production balanced and avoid an excessive metabolite burden on the bacterial cell.

4.4 MATERIALS AND METHODS

4.4.1 BACTERIAL STRAINS AND GROWTH CONDITIONS

Bacterial strains used in this work are listed in Table 4.5. Bacteria were grown at 30°C or 37°C in M9 salts supplemented with 0.5% (w/v) glucose, 0.02% peptone, and 0.01% yeast extract (M9 Glu-sup; see section 2.4.1 page 56). When needed, antibiotics were used at the following concentrations: chloramphenicol, 35 μg mL^{-1}; tetracycline, 25 μg mL^{-1} and ampicillin, 100 μg mL^{-1}. The *E. coli C* mutant derivatives deleted in the *csgA, bcsA, wcaD, pgaA, dos* gene respectively (Table 4.5) were obtained by bacteriophage P1 transduction (Miller 1972).

4.4.2 PNAG DETECTION BY DOT-BLOT ANALYISIS

PNAG detection was carried out essentially as described in Cerca and Jefferson (2008). Briefly, bacteria were grown overnight in 3 mL of M9 Glu-sup medium at 37 °C. The cultures were diluted in Tris-buffered saline [20mM Tris-HCl, 150mM NaCl (pH 7.4)] to produce an $OD_{600\ nm}$= 1.5. Bacteria were collected from 1mL of each suspension by centrifugation, resuspended in 300 μL of 0.5M EDTA (pH 8.0), and incubated for 5 min at 100 °C. Cells were harvested by centrifugation at 10500*g*, 6 min and 100 μL of the supernatant was incubated with 10 μL of proteinase K (20mg μL^{-1}; Sigma Aldrich) for 60min at 60 °C. Proteinase K was heat inactivated for 30 min at 80 °C. This solution was then diluted threefold in of TSB and 100 μL of each dilution were immobilized using the Bio-rad Dot-blot apparatus on a nitrocellulose filter. Blocking of non-specific binding is achieved by placing the membrane in a solution of milk (5%) and TSB, than the nitrocellulose filter is incubated overnight at 4°C with a purified PNAG antibody (diluition 1:500), which is a kind gift of Prof. Gerlad B. Pier (Harvard Medical School, Boston, MA, USA). PNAG antibodies were detected using a secondary anti-goat antibody (diluition 1:5000) conjugated with horseradish peroxidase.

Table 4.5 Bacterial strain and plasmids used in this study.

E. coli strains	Relevant genotype or characteristics	Reference or source
C-1a	*E. coli C* standard laboratory strain F⁻, prototrophic a='Adapted' c-1, *i.e.* maintained for several years on Davis minimal medium. Grows much more rapidly than C-1	Sasaki and Bertani 1965
C-5691	C-1a ∆*pnp*	This thesis
C-1a *ΔcsgA*	C-1a *ΔcsgA::cat*	This thesis
C-1a *ΔbcsA*	C-1a *ΔbcsA::cat*	This thesis
C-1a *ΔwcaD*	C-1a *ΔwcaD::tet*	This thesis
C-1a *ΔpgaA*	C-1a *ΔpgaA::cat*	This thesis
AD01	C-1a *dos::tet*$\Delta_{1200\text{-}2400}$	This thesis
C-5691 *ΔcsgA*	C-1a ∆*pnp ΔcsgA::cat*	This thesis
C-5691 *ΔbcsA*	C-1a ∆*pnp ΔbcsA::cat*	This thesis
C-5691 *ΔwcaD*	C-1a ∆*pnp ΔwcaD::tet*	This thesis
C-5691 *ΔpgaA*	C-1a ∆*pnp ΔpgaA::cat*	This thesis
Plasmids		
pJAMA8	Control vector for luciferase assays, ampicillin resistance	Jaspers *et al.* 2000
pJAMA8-P*pgaA*	*pgaA* promoter and regulatory region (-116 to +234 relative to transcription start site) cloned into the SphI/XbaI sites of pJAMA8	This thesis
pJAMA8-P*pgaA*∆UTR	*pgaA*ΔUTR region (-116 to +23 relative to transcription start site) cloned into the SphI/XbaI sites of pJAMA8	This thesis

Table 4.6 Primers used in this study.

Primers	Sequence	Utilization
16S_for	5'-TGTCGTCAGCTCGTGTCGTGA-3'	qRT-PCR
16S_rev	5'-ATCCCCACCTTCCTCCGGT-3'	qRT-PCR
pgaA_RT_for	5'-CCGCTACCGTCATCAGCAATT-3'	qRT-PCR
pgaA_RT_rev	5'-AGCGCCTTTTGCCACAGTGT-3'	qRT-PCR
glgB_RT_for	5'-TCCGATCGTATCGATAGAGACG-3'	qRT-PCR
glgB_RT_rev	5'-TCGGTTCAATCACCCACACAT-3'	qRT-PCR
speG_RT_for	5'-CCGCTGGAGCGTGAAGATTTA-3'	qRT-PCR
speG_RT_rev	5'-CCGTTCGCTCTGATCGTGAAT-3'	qRT-PCR
fiu_RT_for	5'-TTCGCTCACGTTCTTTGCCG-3'	qRT-PCR
fiu_RT_rev	5'-CGAGAATTTCGGATCGGCAG-3'	qRT-PCR
lptD_RT_for	5'-CATGATTGCCACCGCCCTTT-3'	qRT-PCR
lptD_RT_rev	5'-CTGTACCAGAGGACGGTCAT-3'	qRT-PCR
lptE_RT_for	5'-TGGCATCTGCGTGATACCAC-3'	qRT-PCR
lptE_RT_for	5'-TACGCACCGCACGGCTTAAT-3'	qRT-PCR
cat_rev	5'-GGGCACCAATAACTGCCTTA-3'	Mutant verification
tet_rev	5'-GAAGCTAAATCTTCTTTATC-3'	Mutant verification
wcaD_for	5'-GATATTTGGTACCACGCTC-3'	Mutant verification
bcsA_for	5'-CTAAGCAACCAGTAGGTGAATA-3'	Mutant verification
csgA_for	5'-ACAGTCGCAAATGGCTATTC-3'	Mutant verification
pgaA_for	5'-TGGACACTCTGCTCATCATTT-3'	Mutant verification
pPgaA-delUTR _for	5'- GCATGCAACAATTAAATCCGTGAGT GCCG-3'	*pgaA* promoter cloning
pPgaA-delUTR_rev	5'- TCTAGAATCTTCAGGAATACGGCAT AAAT-3'	*pgaA* promoter cloning
pPgaA_wt_for	5'- AGCATGCCTCAAATAGTCTTTTTCCAT-3'	*pgaA* promoter cloning
pPgaA_wt_rev	5'- ATCTAGATACATCCTGTATTACTCCATG-3'	*pgaA* promoter cloning

4.4.3 PROTEIN LOCALIZATION EXPERIMENTS

Cell fractionation was performed as described in Deflaun *et al.* 1994. Portions (100 mL) of cultures grown in M9 Glu-sup at 37°C for 18 h were centrifuged at 4,000*g* for 10 min at 4°C and washed with 5 mL of 0.1M phosphate buffer pH 7.0 (PB). Cells were resuspended in 2 mL of PB with addition of 100 µg of lysozyme/ml and incubated at room temperature for 30 min. Cells were disintegrated by sonication and centrifuged as described above to remove unbroken cells. The low-speed centrifugation supernatant was then centrifuged at 100,000*g* for 1 h at 4°C to separate the cytoplasm (supernatant) and the membrane fraction (pellet). The pellet was resuspended in 2 mL of 2% Sarkosyl in phosphate buffered saline, left for 20 min at room temperature, and centrifuged at 40,000*g* at 10°C for 10 min to remove ribosomes and cytoplasmic proteins that were still associated with the membrane fraction. The pellet was resuspended in 1 mL of 1% Sarkosyl, precipitated again 20 min at room temperature, and centrifuged as described above. The supernatant, corresponding to inner membrane proteins, was collected, and the pellet, corresponding to outer membrane proteins, was resuspended in 0.2 mL of H_2O. Protein concentrations were determined, and 20 µg of total proteins was loaded onto a 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). Specific bands were identified by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) analysis of the peptide products after in-gel trypsin digestion (performed by CEINGE, University of Naples “Federico II”, Naples, Italy)

4.4.4 OTHER METHODS

Biofilm formation in microtiter plates was determined by the crystal violet staining assay as described in section 2.4.2 page 58. qRT-PCR for determination of *pgaA*, *glgB*, *speG*, *fiu*, *lptD* and expression was performed as described in Gualdi *et al.* (2007), using 16S RNA as reference gene. Luciferase assays were performed as described (Brombacher *et al.* 2003), using the vector pJAMA8 (Jaspers *et al.* 2000), which carries a promoterless *luxAB* genes from *Vibrio harveyi* and resistance to ampicillin. The *pgaABCD* promoter and regulatory region, ranging from -116 to +234 nucleotides relative to the *pgaABCD* mRNA start site, and the *pgaABCD* promoter region in which the untranslated region of the transcript was eliminated (ΔUTR, ranging from -116 to +23 nucleotides relative to the *pgaABCD* mRNA start site) were amplified from the chromosomal DNA using primers including the SphI and the XbaI restriction sites (Table 4.6) and cloned into the multiple cloning site of pJAMA8 to obtain $pPgaA_{WT}$ and $pPgaA_{\Delta UTR}$, respectively.

FINAL REMARKS

In nature bacteria exist in either a planktonic-motile single-cell state or an adhesive multicellular state known as biofilm. Biofilms can cause medical problems and technical damage since they are resistant against antibiotics, disinfectants or the attacks of the host immune system (Costerton *et al.* 1995). Biofilms are characterized by the presence of an extracellular matrix which is mainly composed by extracellular polysaccharides (EPSs) proteins and even (extracellular) DNA. Biofilm formation is subjected to a complex regulation that involves signal molecules: in particular Gram negative bacteria use cyclic diguanylate (c-di-GMP) as a biofilm-promoting second messenger (Hengge 2009). Two classes of enzymes are involved in c-di-GMP metabolism: diguanylate cyclases (DGCs), which synthesize c-di-GMP, and phosphodiesterases (PDEs) that hydrolyze the signal molecule. The enzymes involved in c-di-GMP metabolism are widely conserved in *Eubacteria*, but they are not present in human and, except rare exceptions, in other eukaryotes (Galperin 2004). This makes proteins involved in c-di-GMP metabolism a very interesting target for antimicrobial compounds with anti-biofilm activity. To monitor DGC-activity and to screen for specific inhibitors, I set up a combination of microbiological assays that rely on detection of c-di-GMP-dependent EPS production and biofilm formation. I found that sulfathiazole and azathioprine both known inhibitors of nucleotide biosynthesis inhibit c-di-GMP production and prevent biofilm formation both in laboratory strains and in clinical isolates of *E. coli*. However neither sulfathiazole nor azathioprine showed any inhibition of DGC activity *in vitro* using PleD protein from *C. crescentus.* Thus inhibition of c-di-GMP biosynthesis might take place in an indirect fashion, namely through inhibition of the intracellular nucleotide pool. Indeed, nucleotide starvation could affect the intracellular GTP concentrations which in turn might result in decrease substrate concentrations for DGCs. It is possible that DCGs have relatively low affinity for GTP; indeed, GTP is the substrate for many essential enzymes and, consequently, under nucleotide starvation leading to reduction in intracellular GTP concentration, GTP flux would be directed towards essential metabolism (*e.g.* transcription; translation), becoming unavailable for c-di-GMP biosynthesis. This hypothesis is supported by literature data which strongly suggests that perturbation of intracellular nucleotide pools could indeed interfere with molecular signaling leading to biofilm formation (Ueda *et al.* 2009; Garavaglia *et al.* 2012). Blocking c-di-GMP synthesis in an indirect fashion by blocking GTP supply to DGCs might be an effective strategy and a promising approach to control of biofilm formation (Figure 5.1).

In the second part of my Ph.D. thesis I focused on biofilm regulation. A general scheme resulting from literature data and my findings is shown in Figure 5.1. In particular, my results reiterate the complexity of biofilm regulation and highlight the specific interactions between DCGs and adhesion factors. I show that YddV-Dos complex modulates the production of two adhesion factors namely curli and poly-*N*-acetylglucosamine (PNAG). Adhesion factors' production and, more in general, biofilm formation respond to stress conditions such low temperature, low oxygen

concentrations, low nutrients and others. For instance c-di-GMP mediated biofilm formation only takes place at sub-optimal growth temperature (*e.g.* 30°C) at which various DCG encoding genes are expressed (Sommerfeldt *et al.* 2009). However stress conditions such as nucleotide starvation negatively affect c-di-GMP concentrations as mentioned before; thus different stress conditions can have opposite effects on biofilm development.

Finally during my Ph.D. I focus my attention on the connection between biofilm and the mRNA processing enzyme polynucleotide phosphorylase (PNPase). In particular I found that PNPase is involved in PNAG production. This observations raised the possibility that PNPase, a protein involved in the global mRNA processing, could regulate adhesion factors production in a c-di-GMP dependent fashion through interactions with the YddV-Dos complex (Tuckerman *et al.* 2011). However my results rule out an involvement of YddV in PNPase-dependent regulation of the PNAG biosynthetic operon *pgaABCD*. The mechanism of biofilm regulation by PNPase remains to be identified; more in general, to this date, environmental and physiological cues modulating gene expression regulation at mRNA level remains elusive and will be an important subject for further investigation.

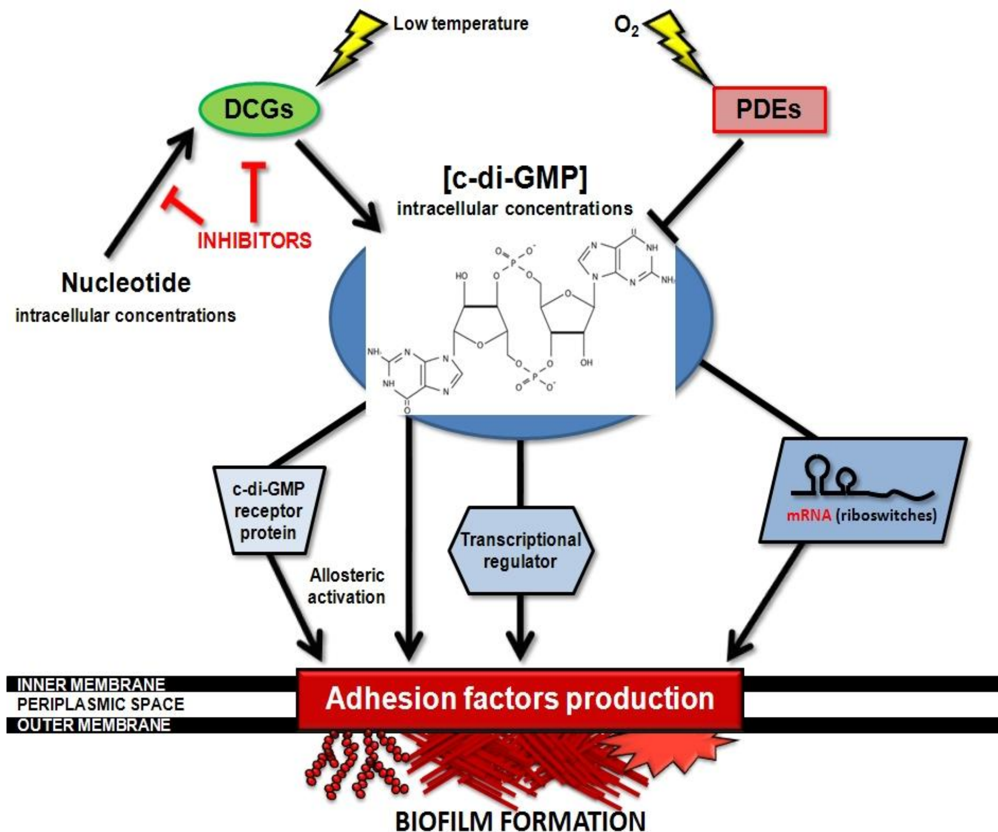


Figure 5.1 Schematic representation of complex c-di-GMP-mediated response in cells.

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