THE SUBTLE BIOFILM REGULATION IN *ESCHERICHIA COLI*: CsgD AND THE *yddV-dos* OPERON

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La gratitudine per il passo compiuto non è solamente
legata alla soddisfazione per aver fatto qualcosa
di importante, ma indica un'esperienza più profonda:
ogni scoperta, piccola o grande, rievoca la percezione di una
misteriosa corrispondenza che lega la realtà all'io umano.
Lasciandosi scoprire e comprendere, la realtà fisica dimostra
di essere fatta per l'io, e l'io conferma la sua vocazione di essere
destinato al rapporto con tutte le cose.
Nell'esperienza della scoperta è come se, per un breve istante,
anche l'apparenza fisica delle cose lasciasse intravvedere
il tratto più vero e ineffabile della realtà:
la segreta amicizia di tutto per l'io umano.

(M. BERSANELLI)

INDEX

1. CHAPTER I: INTRODUCTION

2. CHAPTER II: CsgD REGULATION OF BIOFILM FORMATION

3. CHAPTER III: *yddV-dos* OPERON AND REGULATION OF CURLI FIBERS

4. CHAPTER IV: YddV CONTROLS PRODUCTION OF POLY-*N*-ACETYLGLUCOSAMINE

5. CHAPTER V: STUDY OF PHL1228, A BIOFILM-FORMING MUTANT

INTRODUCTION

INTRODUCTION

1.1 Biofilms

In natural habitats microorganisms are often found attached to solid surfaces (both biotic or abiotic) and organized in structured communities known as biofilms: such communities are characterized by the presence of different bacterial species, often associated with eukaryotic microorganisms, and by the presence of a matrix constituted by extracellular macromolecules (e.g. polysaccharides) which embeds the microbes. The biofilm mode of growth differs significantly from the planktonic state, i.e. bacterial free-living cells (Costerton *et al.*, 1995). The tightly associated cells constituting a bacterial biofilm are able to coordinate their physiological and metabolic state, thus almost resembling the subdivision of functions of a multicellular organism (Caldwell, 2002; Costerton *et al.*, 1995; Shapiro, 1998).

Such "multicellular behavior" offers many advantages to a bacterial population. In the environment, organization into a such community plays a key role in the production and degradation of pollutants and the cycling of nitrogen, sulfur and many metals, carried out by the concerted action of bacteria with different metabolic capabilities. Water channels, throughout the biofilm, provide the exchange of nutrients and metabolism products, allowing metabolic cooperativity (Davey and O'Toole G, 2000). The surrounding extracellular matrix confers a certain degree of protection to the bacteria residing within a biofilm, in particular against biocides and detergents; bacteria growing as biofilm in the human host are less susceptible to the immune response and more resistant to antibiotic treatment (Costerton *et al.*, 1994; Costerton *et al.*, 1999).

Biofilm growth can tremendously impact various human activities: bacterial contaminations, usually by biofilms, can hamper industrial processes, in particular the food and paper industry. 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 (grinding, wash-out with high-pressure water, etc…), but their complete and efficient removal is often difficult.

Biofilm, on the other hand, can also have beneficial functions: for instance, removal of organic material and more efficient processing of contaminants in wastewater treatment, or bioremediation of polluted soil, are mostly carried out by biofilm-growing bacteria.

Importance of biofilms in bacterial infections

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. 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 *E. coli*) infections are mainly due to biofilms, and show remarkable resistance to antibiotic treatment. This resistance results in establishment of chronic bacterial infections (Finlay and Falkow, 1997; Hoyle and Costerton, 1991).

Production of exopolysaccharides can protect the bacterial cell against the immune response of the host (Vuong *et al.*, 2004), and can be stimulated by exposure to antibiotics. Thus, antibiotic treatment can be counterproductive in some cases and even promote biofilm formation (Rachid *et al.*, 2000; Sailer *et al.*, 2003). An example of extensive colonization linked to exopolysaccharide production are lung infections caused by *Pseudomonas aeruginosa*, often in concert with other opportunistic pathogens. In cystic fibrosis (an inherited chronic disease that affects the lungs and digestive system) patients, infections by *P. aeruginosa* are the main cause of death. Gene transfer is enhanced within biofilm (Ghigo, 2001; Li *et al.,* 2001; Molin & Tolker-Nielsen, 2003), thus providing for quick and efficient transfer of antibiotic resistance genes, and making the combat of biofilm-born infections difficult. Moreover, biofilms may consist of cells of several or a single bacterial species interacting cooperatively. Cells within a biofilm are physiologically heterogenous because of a variety of microniches occur within the biofilm structure. Cells on the surface of the structure with ready access to nutrients would be metabolically active and able

to divide; in contrast cells in the internal layers of a biofilm may be largely dormant. This concept of physiological heterogeneity within a biofilm is important because, unlike the relative physiological synchrony of planktonic bacteria in broth culture, biofilm heterogeneity results in cells with vastly different properties, such as susceptibilities to antibiotics. Indeed, cells in the deeper layers of thick biofilms, which grow more slowly, have less access to antibiotics and nutrients. As a result, biofilms may be as much as 1000 times more refractory to antibiotic killing than bacterial cells suspended in broth culture (Behlau & Gilmore, 2008).

Biofilm development

Transition from the planktonic mode of growth to more a complex structure such as a biofilm occurs as a sequential and developmental process (Fig. 1) (Reisner *et al.*, 2003; Stoodley *et al.*, 2002; Ghigo, 2001). 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 (Jucker *et al.*, 1996; Van Loosdrecht *et al.*, 1990). 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; Otto & Silhavy, 2002; Sauer & Camper, 2001; Wang *et al.*, 2004b). In the presence of environmental conditions allowing bacterial growth, adherent cells can divide and form an attached monolayer known as a microcolony. Establishment of stronger cell-cell contacts allows the microcolony to finally differentiate into a mature biofilm whose three-dimensional structure is determined by the extracellular polymeric substances (EPS) in which the biofilm is encased. EPS is constituted by a highly hydrate milieu consisting of different types of exopolysaccharides, extracellular proteins and enzymes, and even DNA (Lawrence *et al.*, 1991; Withchurch *et al.*, 2002); in addition, bacterial outer membrane vesicles, flagella, phages, pili, host matrix material, and lysed cell debris may also be present (Hunter and Beveridge, 2005). This EPS 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, pores, and even a redistribution of the bacteria farther away from the substratum. Growth generally occurs as the result of binary division of attached cells spreading daughter cells upward and outward from the attached surface to form cell clusters (Heydorn *et al*., 2000); however, recruitment of cells from the bulk fluid to the aggregating microorganisms has also been observed (Molin *et al*., 2000). A mature biofilm can reach a thickness ranging from μm to cm, thus representing a heterogeneous micro-environment with respect to physical and chemical conditions such as osmolarity, oxygen, pH and nutrients (Stoodley *et al.*, 2002).

http://www.pasteur.fr/recherche/RAR/RAR2006/Ggb-en.html
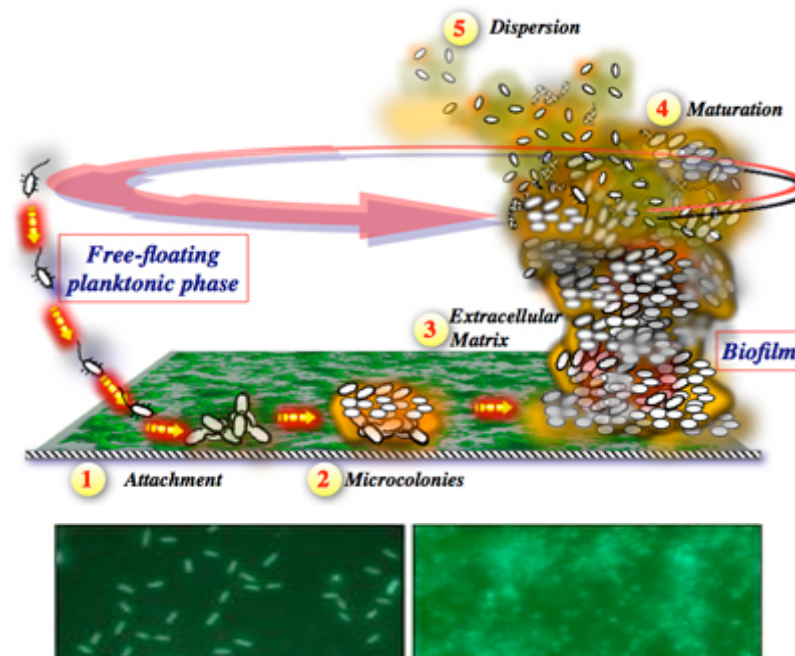
Figure 1. Construction of a biofilm. Free (planktonic) bacteria assemble on a surface.

After biofilm maturation, EPS matrix levels appear to decrease, perhaps due to metabolism, with subsequent detachment (Fig. 1, stage 5) of clumps and individual cells (Gilbert *et al.*, 1998). This detachment event 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 (Jones *et al.*, 1998; Landini *et al.*, 2010).

1.2 Determinants in biofilm formation

Transition from free-living cells to the biofilm mode of growth implies substantial modifications regarding morphology and biochemistry of the cell (Davies *et al.*, 1993; Pratt & Kolter, 1999; Prigent-Combaret & Lejeune, 1999; Prigent-Combaret *et al.*, 1999; Schembri *et al.*, 2003). Thus far several features taking part in biofilm formation have been identified; most of these biofilm determinants are cell-surface exposed or extracellular structures directly involved in attachment to substrata and in cell aggregation.

In the following parts, I will give a general overview of the different components involved in adhesion and biofilm formation, and then I will focus in particular on two determinants: curli fibers, a structural determinant in *E. coli*, and the recent discovered class of the GGDEF-EAL proteins; both curli and GGDEF proteins are subjects of my experimental work.

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.*, 1997). In *Pseudomonas* B13 variations in the composition of external LPS appear to be responsible for the different adhesion behavior of at least three bacterial subpopulations (Simoni *et al.*, 1998).

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 Vibrio cholerae* mutants are not able to form biofilm (Nesper *et al.*, 2001). However, the loss of the adhesion properties seem to be caused by affected type I fimbriae and/or flagella (see below), associated with these mutations, rather than to 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 & 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 (Rijnaarts, 1994; van Loosdrecht *et al.*, 1990).

Flagella

Flagella are filamentous structures, mainly constituted by the flagellin protein, the product of the *fliC* gene. Flagella allow bacterial motility and play a central role in chemotaxis. Their role in adhesion processes might be to overcome surface repulsion and to stabilize surface contact. Mutations affecting *E. coli* flagellar functions, such as *flh* or *mot*, abolish both initial interaction with the surface and movement along the surface (Danese *et al.*, 2000a; Pratt & Kolter, 1998). A functional flagellum has been suggested to be involved in biofilm formation by *Pseudomonas sp.* as well, since insertion mutation in either *flgK* or *fliP*, both involved in flagellar synthesis, lead to non-motile strains deficient in biofilm formation (O'Toole & Kolter, 1998a; O'Toole & Kolter, 1998b). In contrast, motility is not essential to biofilm formation in curli-producing *E. coli* strains (Prigent-Combaret *et al.*, 2000) or in *Pseudomonas fluorescens* biofilm-forming, non-motile mutants (Robleto *et al.*, 2003). Presence of flagella in *P. putida* was found in planktonic cultures prior to biofilm formation, and again in mature biofilms, but not during the microcolony state, suggesting a role of flagella in initiation of cell-surface contact as well as in the detachment of cells from the biofilm (Sauer & Camper, 2001).

Pili and Fimbriae

Like flagella, pili and fimbriae are extracellular structures constituted by proteins which, however, are not involved in cell motility. Type I fimbriae are short and numerous, and are encoded by the *fim* genes and expressed in most *E. coli* and *Salmonella* strains. 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 (Boddicker *et al.*, 2002; Pratt & Kolter, 1998; Schembri *et al.*,

2001). 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.*, 2002). Ag43 mediates cell-cell or cell-surface contacts and promotes biofilm formation in glucose-minimal medium in *E. coli* (Danese *et al.*, 2000a). 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 sp*. 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 & Kolter, 1998a). 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 & 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, *Vibrio cholerae* expresses TCP (toxin-coregulated pilus, belonging to the type IV pili group) in the host intestinal surface, where it is an essential colonization factor, whereas attachment to abiotic surfaces such as borosilicate is rather mediated by the mannose-sensitive hemagglutin (MSHA) pilus (Watnick *et al.*, 1999).

Exopolysaccharides

Secreted polysaccharides 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 interfaces, pellicles at air–liquid interfaces, cell aggregates and clumps in liquid cultures, and wrinkled colony morphology on agar plates. Evidence for a structural role of some of these matrix polysaccharides is accumulating, and the regulation of production of these exopolysaccharides is now actively being investigated in different bacteria (Kirillina *et al.* 2004; Branda *et al.*, 2005; Simm *et al.,* 2005). For instance, production of extracellular polysaccharides (EPS), such as alginate in *P. fluorescens* and colanic acid (CA) in *E. coli* is induced after attachment to a solid surface (Davies & 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.*, 2000a; Prigent-Combaret *et al.*, 2000). Same results were found regarding alginate expression of *Pseudomonas aeruginosa* (Wozniak *et al.*, 2003). Exopolysaccharides can remain attached to the cell surface in a capsular form or be released as slime. EPS display a considerable heterogeneity, ranging from simple α,1-4-linked, un-branched glucose polymer called dextran, to the more complex substituted hetero-polysaccharides made up of combinations of different repeating subunits, such as xanthan and colanic acid. EPS biosynthesis is extremely complex: colanic acid synthesis in *E. coli* 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). CA is critical for biofilm three-dimensional structure formation based on the research of Danese and colleagues (2000b), nevertheless, overproduction of colanic acid inhibits biofilm formation in *E. coli* BW25113 strains (Zhang *et al.*, 2008).

In Gram positive *Staphylococcus epidermidis,* expression of the *icaABCD* operon leads to production of polysaccharide intracellular adhesin (PIA), an important factor in colonization of medical devices and in cell-cell adhesion (Conlon *et al.*, 2002; Heilmann *et al.*, 1996; McKenney *et al.*, 1998). PIA production actively protects the bacteria against major components of the human immune system, such as leukocytes and antibacterial peptides (Vuong

et al., 2004). *icaA* is a glycosyltransferase which catalyses the assembly of large polymers of N-acetylglucosamine residues (Gerke *et al.*, 1998; Heilmann *et al.*, 1996). Also other *Staphylococcus* species, such as *S. aureus* and *S. caprae*, were found to form biofilms by *icaABCD*-dependent PIA production (Allignet *et al.*, 2001; Cramton *et al.*, 1999). It appears that *ica*-like genes encode proteins responsible for the production of extracellular polymeric substance (EPS) in a widely distributed group of bacteria. Homologous genes responsible for biofilm formation are found in *Yersinia pestis* and also *E. coli* (see chapter III, IV, V; Darby *et al*., 2002; Joshua *et al.*, 2003; Wang *et al.*, 2004a).

Cellulose is an extracellular matrix component originally identified as an additional determinant for biofilm formation in enterobacteria (Zogaj *et al.*, 2003). Cellulose is present in the biofilm of an *Enterobacter sakazakii* clinical isolate (Grimm *et al.*, 2008). In *Salmonella* strains, a mutant in cellulose production retained some capability to form cell aggregates, but not a confluent biofilm (Jonas *et al.*, 2007). However, the role of cellulose as an adhesion factor is not straightforward (Wang *et al.*, 2006).

Outer membrane proteins (OMPs)

Proteins located in the outer membrane of Gram negative bacteria are often involved in cell-surface attachment. The effects of OMPs are probably less involved in direct interaction, rather than in modulating the effects of surface structures. 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* led 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 & Hermansson, 2004). Thus, OmpX likely affects regulation and/or cell localization of different cell surface structures.

Extracellular DNA

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 *Pseudomonas 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 (Whitchurch *et al.*, 2002). In addition to the structural role of eDNA, intracellular levels of cytidine influence extracellular polysaccharides biosynthesis and surface attachment in *Vibrio cholerae*, thus suggesting that nucleosides might act as signals for biofilm formation (Haugo & Watnick, 2002). Finally, *Streptococcus* mutants defective in competence genes were found attenuated in biofilm formation (Li *et al.*, 2002; 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 for biofilm determinants often requires a combination of different environmental signals, which can modulate the activity of complex regulatory networks or both specific and global regulator. Interestingly, despite the striking biofilm phenotype, only a few biofilm-specific genes and small parts of biofilm-dedicated pathways have been revealed thus far (Ghigò, 2003; Landini, 2009). Adhesion and/or aggregative cellular factors can be part of environmental stress regulons (i.e nutritional or oxidative stress), and so can directly affect transition from single cells to biofilm, biofilm maintenance and even dispersal.

Transcriptional regulation responding to environmental factors.
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 (Gerstel & Romling, 2001; Olsen *et al.*, 1993). Temperature regulation also plays a role in the expression of outer biofilm determinants, such as the *Yersinia pestis hms*

genes, responsible for PIA production: these genes are degraded upon a temperature shift from 26°C to 34°C (Perry *et al.*, 2004). The presence of a specific substrate may trigger opposite reaction in different bacterial species. For instance, biofilm formation by *E. coli* K12, *S. aureus* and *Streptococcus mutans* is repressed by the presence of glucose (Jackson *et al.*, 2002; Regassa *et al.*, 1992; 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 & Tielen, 2010). In contrast, growth in oxygen-limited conditions results in a sharp decrease in *E. coli* adhesion to hydrophilic substrates (Landini & Zehnder, 2002).

Influence of environmental factors can be mediated by two-component regulatory systems that can sense the changes in the environmental conditions and trigger a specific cellular response. Two-component regulatory systems are constituted by a sensor protein, usually found in the membrane, and by a regulatory protein, able to bind specific sequences on the DNA. Transcription regulation is triggered by chemical modification of an inactive regulatory protein (usually by phosphorylation) carried out by the sensor protein. Several two-component regulatory systems 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. 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. A CpxR mutant strain forms less stable cell-surface interactions in comparison to the wild type strain (Otto & Silhavy, 2002). Consistent with this, when *E. coli* cells interact with a hydrophobic surface, the Cpx pathway is activated (Otto & 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, another two-component signalling pathway, appears to have a role in promoting stable cell–surface interactions in response to increased osmolarity (Otto & Silhavy, 2002). A strain of *E. coli* with a mutation in the OmpR protein (OmpR234) 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 two-component systems 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).

Intracellular signal molecules

Products of amino acids degradation may function as intracellular signal molecules involved in adhesion. Indole has a role on biofilm formation. Indole production is a phenotypic trait displayed by several Gram-negative bacteria including *E. coli* and it is produced by the degradation of tryptophane, a reaction performed by tryptophanase encoded by the *tnaA* gene (Newton and Snell, 1964). Indole has been described as a potential extracellular signal (Wang *et al.*, 2001). Genes necessary for indole production (including *tnaA*) have been shown to be induced by addition of *E. coli* stationary phase supernatant (Ren *et al.*, 2004), suggesting the existence of complex cross-talk between different extracellular signaling pathways. A mutant of *E. coli* K-12 S17-1 for gene *tnaA* is unable to produce a biofilm (Di Martino *et al.*, 2002). Another diffusible molecule, O-acetyl-l-serine (OAS), appears to modulate *E. coli* biofilm formation. A mutation in the gene coding for a serine acetyltransferase *cysE*, which catalyzes the conversion of serine to O-acetyl-l-serine, was shown to enhance biofilm formation through reduction of the amount of an extracellular signal molecule. The authors suggest that OAS or other cysteine metabolites may play a physiological role, possibly by activating genes whose expression leads to inhibition of biofilm formation (Sturgill *et al.*, 2004).

Medicago sativa produces L-canavanine (a nonproteinogenic amino acid, structurally related with the proteinogenic L-arginine) (Plaga *et al.*, 1998) that interferes with the quorum sensing system of its nitrogen-fixing symbiont *Ensifer meliloti* and inhibits the expression of genes for the production of exopolysaccharides in the bacterium. Moreover, recently Kolodkin-Gal *et al.*, 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).

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 Fuqua *et al*. (1994), is an example of cell-to-cell communication and depends on small, diffusible signal molecules called autoinducers (Kaplan & Greenberg, 1985). 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 level), it induces phenotypic effects by regulating QS-dependent target gene expression. Bacterial QS mechanism is based on two groups of signal molecules: peptide derivatives typical for Gram-positive bacteria, and fatty acid derivatives typical for Gram-negative bacteria.

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 (Teplitsky *et al.*, 2003; Fuqua *et al.*, 1994), which are utilized by the bacteria to monitor their own population. 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 (Eberl, 1999). QS regulates several events in *P. aeruginosa* beside biofilm formation, such as the production of virulence factors and

secondary metabolites, as well as the adaptation and survival in stationary phase. 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 structures, rather than for the first steps of biofilm formation. It is worth mentioning that QS signals in *P. aeruginosa* biofilm are detectable in lungs of cystic fibrosis patients (Singh *et al.*, 2000), suggesting that QS might be a possible target for preventing biofilm formation. Quorum sensing in *Escherichia coli* and *Salmonella* has been an elusive topic for a long time. However, in the past 8 years, several research groups have demonstrated that these bacteria use several quorum-sensing systems, such as: the luxS/AI-2, AI-3/epinephrine/norepinephrine, indole, and the LuxR homolog SdiA to achieve intercellular signaling. The majority of these signaling systems are involved in interspecies communication, and the AI-3/epinephrine/norepinephrine signaling system is also involved in interkingdom communication. Both *E. coli* and *Salmonella* reside in the human intestine, which is the largest and most complex environmental niche in the mammalian host. The observation that these bacteria evolved quorum-sensing systems primarily involved in interspecies communication may constitute an adaptation to this environment (Walters & Sperandio, 2006).

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 has been shown to 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 flagella, type I fimbriae, LPS, and colanic acid, most of them linked to environmental stimuli

including pH, oxygen, temperature, and osmolarity (Dorman & Bhriain, 1992; Olsen *et al.*, 1998; Hommais *et al.*, 2001; Dorman, 2004). The H-NS protein appears to have a direct relationship with biofilm formation: it is able to inhibit formation of complexes between promoters and σ^{70}-RNA polymerase (the main form of RNA polymerase during the exponential phase of growth). However, RNA polymerase associated with σ^s, an alternative σ factor mainly active in stationary phase, can by-pass H-NS inhibition. This effect 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, 1994a). 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 & 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 seems to directly affect biofilm formation, though its role is still controversial. It governs the expression of many genes induced during the stationary phase of growth and in *P. aeruginosa* the QS system appears to be related to RpoS expression 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 & McLean, 1999). Schembri *et al*. found that 46% of RpoS-dependent genes to be differently expressed in biofilms and deletion of *rpoS* rendered *E. coli* incapable of establishing sessile communities (Schembri *et al.*, 2003). 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.

sRNA and biofilm regulation

In bacteria, more than 150 non-coding small RNAs (sRNAs) have been described (Livny & Waldor, 2007). The first bacterial sRNAs were discovered in *Escherichia coli*, either fortuitously due to their abundance or by the observation of phenotypes conferred by their overexpression. Most bacterial sRNAs affect gene expression regulation, usually at the 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 the mRNA stability. A second type of post-transcriptionally active sRNAs interacts with RNA-binding regulatory proteins of the RsmA/CsrA family. RsmA (regulator of secondary metabolism) and CsrA (carbon storage regulator) can act as translational repressors; sRNAs having high affinity for these proteins are therefore able to relieve translational repression by sequestering them (Babitzke & Romeo, 2007). Recently, it has been discovered that a lot of these sRNA are involved in the expression regulation of biofilm formation. For instance, CsrA, a global regulator involved in the control of motility and biofilm formation as well as in virulence and pathogenesis, quorum sensing and oxidative stress response, is regulated at the post-transcriptional level by two sRNAs called CsrB and CsrC in *E. coli* (Dubey & Babitzke, 2005; Liu *et al.*, 1997). These sRNAs are composed of multiple CsrA binding sites that bind and sequester CsrA, thereby inhibiting its activity. 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).

GGDEF and EAL proteins: c-di-GMP turnover

A class of bacterial proteins containing a so-called GGDEF/EAL domain appears to play a central role in sensing environmental conditions and in directing bacterial response to a molecular level. A significant number of these GGDEF/EAL proteins are directly involved in the expression of virulence and/or biofilm factors through c-di-GMP production hydrolysis. Bis-(3´-5´)-cyclic dimeric guanosine monophosphate (c-di-GMP) was discovered by Benziman and co-workers as a factor that allosterically activates the membrane bound cellulose synthase of *Gluconacetobacter xylinus* (Ross *et al.*, 1987; Tal *et al.*,

1998). c-di-GMP is a soluble molecule that functions as a second messenger in bacteria. In general, c-di-GMP stimulates the biosynthesis of adhesins and exopolysaccharide matrix substances in biofilms and inhibits various forms of motility: it controls switching between the motile planktonic and sedentary biofilm-associated 'lifestyles' of bacteria. Moreover, c-di-GMP controls the virulence of animal and plant pathogens (Cotter & Stibitz, 2007; Ryan *et al.*, 2007; Tamayo *et al.*, 2007), progression through the cell cycle (Duerig *et al.*, 2009), antibiotic production (Fineran *et al.*, 2007) and other cellular functions. c-di-GMP is produced from two molecules of GTP by diguanylate cyclases (DGCs) and is broken down into 5'-phosphoguanylyl-(3'-5')-guanosine (pGpG) by specific phosphodiesterases (PDEs); pGpG is subsequently split into two GMP molecules (Fig. 2) by EAL phosphodiesterases albeit with a much slower kinetic (Schmidt *et al.*, 2005), or by other cellular enzymes.

DGC activity is associated with the GGDEF domain, which is named after the amino acid sequence motif that is an essential part of the active site of the enzyme (Malone *et al.*, 2007; Chan *et al*., 2004). c-di-GMP-specific PDE activity is associated with the EAL or HD-GYP domains; these amino acid motifs of both domains are essential for their enzymatic activities (Christen *et al.*, 2005; Schmidt *et al.*, 2005; Ryan *et al.*, 2006; Hengge, 2009). The active DGC is a dimer of two subunits with GGDEF domains. The active site (A site) is located at the interface between the two subunits, which each bind one molecule of GTP (Chan *et al.*, 2004; Christen *et al.*, 2005).
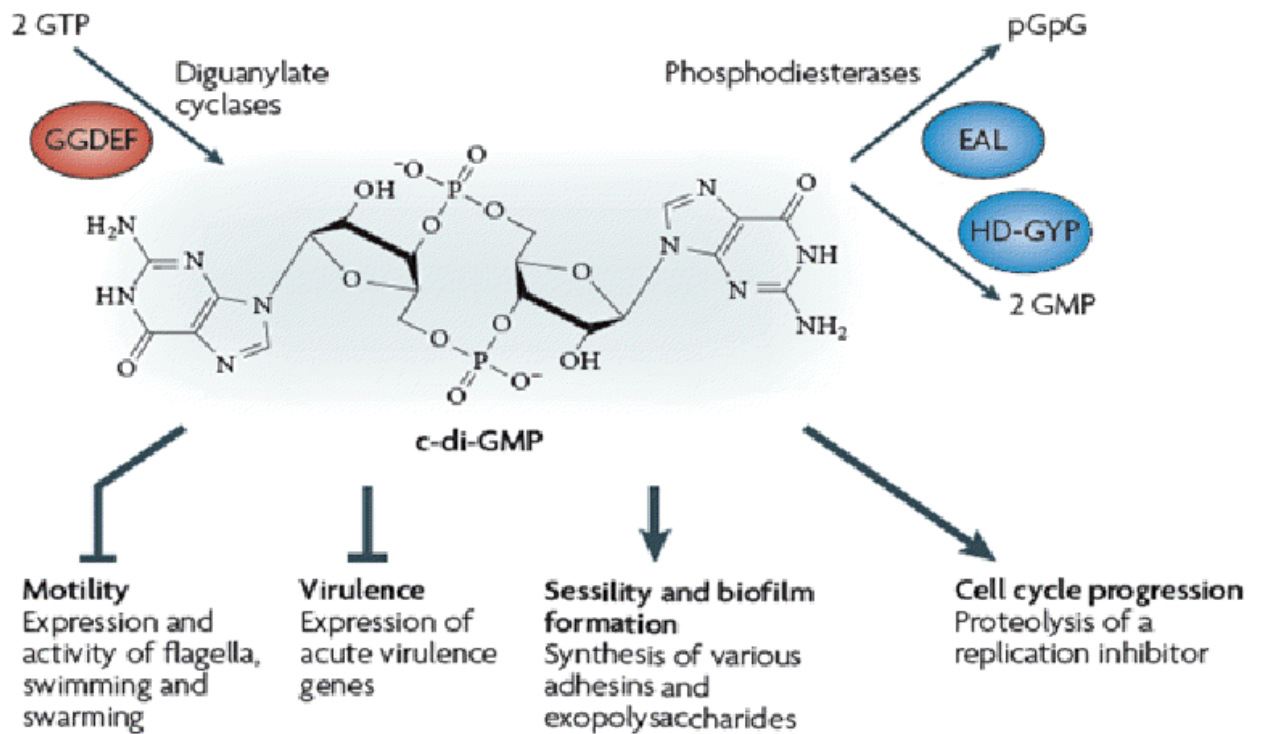
R. Hengge

Figure 2. **Structure and functions of c-di-GMP**. At the cellular level, bis-(3'-5')-cyclic dimer guanosine monophosphate (c-di-GMP) levels are controlled by GGDEF (red) and EAL (blue) proteins. c-di-GMP 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 and matrix exopolysaccharides. pGpG, 5'-phosphoguanylyl-(3'-5')-guanosine.

The A site corresponds to the GGDEF motif, and any point mutation in this motif (except a D to E mutation) eliminates enzymatic activity. An active EAL domain PDE is a monomeric enzyme that linearizes c-di-GMP to 5'-pGpG, which is then further degraded by non specific cellular PDEs. The second type of c-di-GMP-specific PDE is the HD-GYP domain proteins, which form a subfamily of the HD superfamily of metal-dependent phosphohydrolases and are unrelated to the EAL proteins (Galperin *et al.*, 1999). These enzymes break the phosphodiester bond in c-di-GMP to produce 5´-pGpG, and can further degrade 5´-pGpG to GMP.

Escherichia coli GGDEF and EAL proteins

E. coli K-12 has 29 genes involved in c-di-GMP turnover, which encode 12 proteins with GGDEF domains, 10 proteins with EAL domains and seven

proteins that feature both domains. Given these numbers, it is easy to imagine that target components or processes controlled by these effectors are various and different. However, c-di-GMP physiological impact nor its effector mechanisms are still poorly understood. In general, functions that contribute to biofilm formation, are positively regulated by c-di-GMP, while motility is downregulated by c-di-GMP, as can be show by overproducing GGDEF or EAL proteins (D'Argenio *et al.*, 2002; Simm *et al.*, 2004; Tischler and Camilli, 2004; Lim *et al.*, 2006). At least two separate DGC–PDE systems (YdaM–YciR and YegE–YhjH) control the transcription of the *csgDEFG* operon, which encodes the transcriptional regulator CsgD and several proteins required for curli assembly and export. Moreover, the DGC AdrA (also referred to 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 (Brombacher *et al.*, 2006). 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.

CsgD REGULATION OF BIOFILM FORMATION

INTRODUCTION

The experiments described in this chapter have been published in the following publications:

- **Gualdi, L., Tagliabue, L. & Landini, P. (2007).** Biofilm Formation-Gene Expression Relay System in *Escherichia coli*: Modulation of σ^S-Dependent Gene Expression by the CsgD. *J. Bacteriol.* **189**: 8034-8043.

- **Gualdi, L., Tagliabue, L., Bertagnoli, S., Ieranò, T., De Castro, C. & Landini, P. (2008).** Cellulose modulates biofilm formation by counteracting curli-mediated colonization of solid surfaces in *Escherichia coli*. *Microbiology.* **154**: 2017-2024.

In enterobacteria, curli fibers (also known as Tafi, thin aggregative fimbriae, in *Salmonella*) are an important factor in adhesion to surfaces, cell aggregation, and biofilm formation (Doran *et al.*, 1996; Olsen *et al.*, 1993; Romling *et al.*, 1998a). Curli-encoding genes are clustered in two operons: *csgBA* encodes the structural components, while the divergently oriented *csgDEFG* operon encodes proteins involved in curli assembly and transport, as well the CsgD transcription factor, which is necessary for *csgBA* transcription (Arnqvist *et al.*, 1994; Hammar *et al.*, 1995). Expression of the *csg* operons takes place in response to a combination of environmental conditions, such as slow growth rate (<32°C), low osmolarity, and slow growth (Olsen *et al.*, 1993). Such environmental signals are mediated at the gene expression level by a number of regulators, including, besides CsgD, global regulatory proteins such as OmpR, H-NS, CpxR, and the alternative σ factor σ^s (Arnqvist *et al.*, 1994; Romling *et al.*, 1998; Prigent-Combaret *et al.*, 2001; Bougdour *et al.*, 2004). However, this strict environmental control is lost in several bacterial strains due to mutations either in regulatory genes (Arnqvist *et al.*, 1994; Vidal *et al.*, 1998) or in the *csgDEFG* sequence (Romling *et al.*, 1998a). Indeed, temperature-dependent regulation does not take place in several *Salmonella* and pathogenic *E. coli* strains, in which curli are also expressed at 37°C and represent an important

virulence factor (Ben *et al.*, 1996; Bian *et al.*, 2000; Persson *et al.*, 2003). In contrast, curli operons are silent in a large number of laboratory strains, as well as in some clinical and environmental isolates, despite the presence of functional *csg* genes (Romling *et al.*, 1998).

In addition to its role as activator of the *csgBA* operon, CsgD activates cellulose production, which results in the formation of a curli/cellulose extracellular matrix (Romling *et al.*, 2000; Zogaj *et al.*, 2001). CsgD stimulates cellulose production indirectly, by activating transcription of the *adrA* gene; in turn, the AdrA protein positively affects the enzymatic activity of the cellulose biosynthetic machinery through its diguanylate cyclase activity, i.e. synthesis of the signal molecule c-di-GMP. Although cellulose was originally described as an additional determinant for biofilm formation in enterobacteria (Romling *et al.*, 2000), its role as an adhesion factor is not straightforward (Wang *et al.*, 2006).

This part of my work describes the effects of CsgD overexpression on biofilm regulation and global gene expression in MG1655, a well-characterized laboratory strain used as standard reference for *E. coli* (Blattner *et al.*, 1997).

RESULTS

Part I

As mentioned in the previous section, several laboratory strains, including MG1655, are only capable to produce curli fibers in limited amounts, insufficient to promote biofilm formation in standard laboratory assays (Fig. 1, WT/pT7-7, left panel), even though the genes necessary for curli biosynthesis and assembly are fully functional (Romling *et al.*, 1998). However, albeit low, curli production takes place at sufficient levels to be determined on agar medium supplemented with Congo Red stain (CR medium). CR is a dye able to bind to exopolysaccharides and amyloid fibers; thus, due to their ß-amyloid structure, curli bind CR with very high affinity.
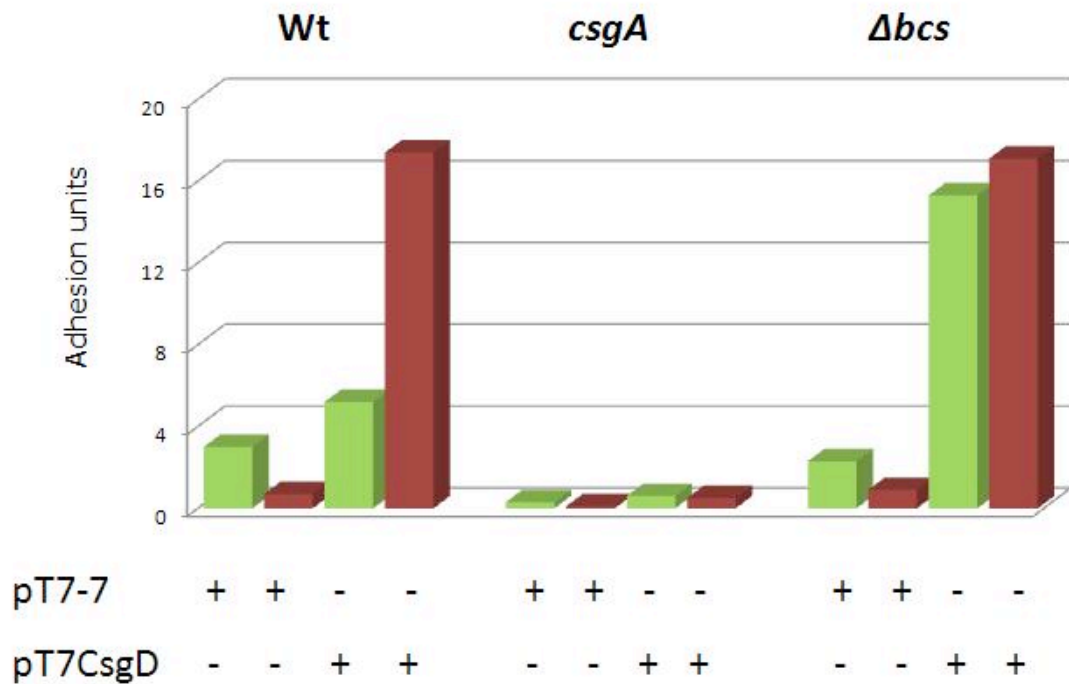
Figure 1: Biofilm formation on microtiter plates of *E. coli* MG1655 (wild-type, WT), PHL856 (*csgA*::*kan*) and LG26 (Δ*bcs::kan*) transformed with either pT7-7 or pT7CsgD, grown at 30°C (green bars) or at 37°C (red bars). Surface-attached cells were quantified by spectrophotometric determination after CV staining and resuspension in ethanol. Values are the mean of four independent experiments, with a standard deviation always lower than 10%.

Because of MG1655 low ability to adhere to a surface, we overexpressed CsgD in this strain in order to boost biofilm formation. In the pT7CsgD plasmid, the *csgD* gene is under the control of a promoter dependent on the bacteriophage T7 RNA polymerase. In *E. coli* strains such as MG1655, which do not carry the T7 RNA polymerase-encoding gene, low-level, constitutive transcription of genes under the control of pT7 promoters can still take place, due to weak promoter recognition by bacterial RNA polymerase.

Role of cellulose on biofilm formation. In *E. coli*, expression of the *csgBA* genes, leading in turn to curli production and biofilm formation, is temperature-regulated and can take place at ≤30 °C and not at 37 °C (Olsen *et al.*, 1993; Romling *et al.*, 1998a; Prigent-Combaret *et al.*, 2001; Castonguay *et al.*, 2006). However, transformation of MG1655 with pT7CsgD results in dramatically stronger biofilm formation at 37 °C compared to 30 °C (Fig. 1). Biofilm formation at 37 °C is totally dependent on a functional *csgA* gene,

suggesting that it is still mediated by curli production (Fig. 1). However, *csgBA* operon transcription levels at 30 °C and 37 °C are comparable in MG1655 pT7CsgD (Table 1), suggesting that increased biofilm formation at 37 °C is not mediated by a corresponding increase in curli production.

Table 1. Relative expression of curli- and cellulose-related genes

Strain	*csgB*	*csgD*	*csgG*	*adrA*
MG1655/pT7-7 30°C	1*	1*	1*	1*
MG1655/pT7-7 37°C	0,014	0,11	0,032	0,42
MG1655/pT7CsgD 30°C	2430	60,8	15,3	337
MG1655/pT7CsgD 37°C	1518	33,5	2,25	1,23

*ΔCt between the gene of interest and the 16S gene was arbitrarily set at 1 for MG1655/pT7-7 grown at 30 °C and expressed as relative values for other strains and growth conditions.

These observations strongly suggest that curli can synergize with another factor that is only expressed at 37 °C. To identify such a factor, we analyzed the membrane expression pattern and we determined production of extracellular polysaccharides (EPS) in MG1655/pT7CsgD, both at 30 °C and at 37 °C. While the membrane protein pattern failed to show any significant difference in CsgD-dependent protein expression at both growth temperatures (data not shown), analysis of EPS production showed a high increase in cellulose production when the strain was transformed with the pT7CsgD vector only at 30 °C (Fig. 2). Interestingly, as shown in Fig. 2, increase in cellulose production is not detectable at 37 °C, i.e. in the conditions in which stimulation of biofilm by CsgD is greater. This observation was surprisingly, because cellulose was proposed to be an adhesion factor in *E. coli* (Romling *et al.*, 2000).
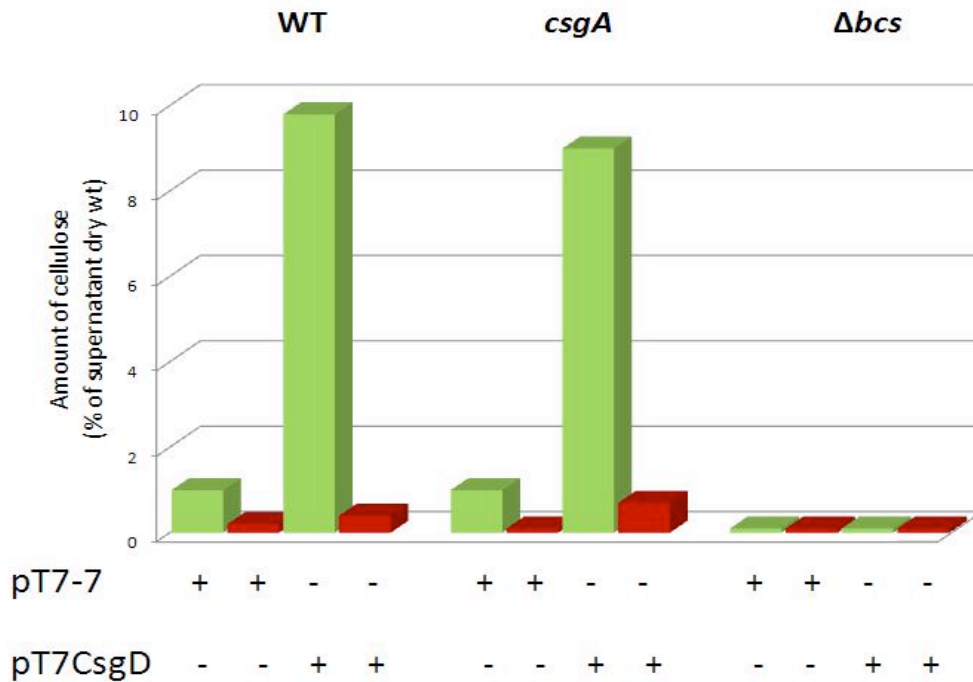
Figure 2. Determination of cellulose production in *E. coli* MG1655 (WT), PHL856 (*csgA*::*kan*) and LG26 (*Δbcs*::*kan*) transformed with either pT7-7 or pT7CsgD, grown at 30 °C (green bars) or at 37 °C (red bars). The values indicate the amount of cellulose as percentage of total dry weight of the culture surpernatant after lyophilization. Values are the mean of two independent experiments with very similar results.

Thus, taking together, these data indicate that, while *csg* genes expression is similar at 30 °C and 37 °C, CsgD-dependent biofilm formation is dramatically higher at 37 °C, the conditions of low cellulose production. These results suggest that cellulose production negatively correlates with adhesion in MG1655 pT7CsgD. Cellulose production was not affected by *csgA* inactivation, while, as expected, was totally abolished in LG26, the *bcsA* mutant derivative of MG1655 (Fig. 2).

To test the hypothesis that cellulose might act as a negative determinant for biofilm formation, we set up adhesion assays with LG26 strain. In contrast with *csgA* inactivation, that abolishes surface attachment by MG1655 even with overexpression of CsgD, deletion of *bcsA* gene has very little effect on MG1655, but it strongly stimulates adhesion to microtitre plates at 30 °C in the presence of pT7CsgD, suggesting that cellulose can negatively affect curli-mediated biofilm formation (Fig. 1). At 37 °C, *bcsA* deletion has very little effect on adhesion, consistent with lack of cellulose production at this temperature.

What is the negative role of cellulose on biofilm formation? Considering that cellulose production does not inhibit curli-dependent biofilm formation through negative regulation of curli-encoding genes (Table 1), we decided to test cellulose effects on Congo red staining. MG1655 colonies are red on CR medium, due to curli production; the red phenotype is totally abolished by inactivation of the *csgA* gene, encoding the major subunit of curli, but is not affected by a mutation in the *bcsA* gene, responsible for cellulose production (Fig. 3). Moreover, lack of cellulose production at 37 °C is able to restore the red phenotype in the presence of the pT7CsgD plasmid (Fig. 3). So, it seems to be that high cellulose production would shield binding of curli fibers to CR, thus leading to less curli-dependent biofilm formation.
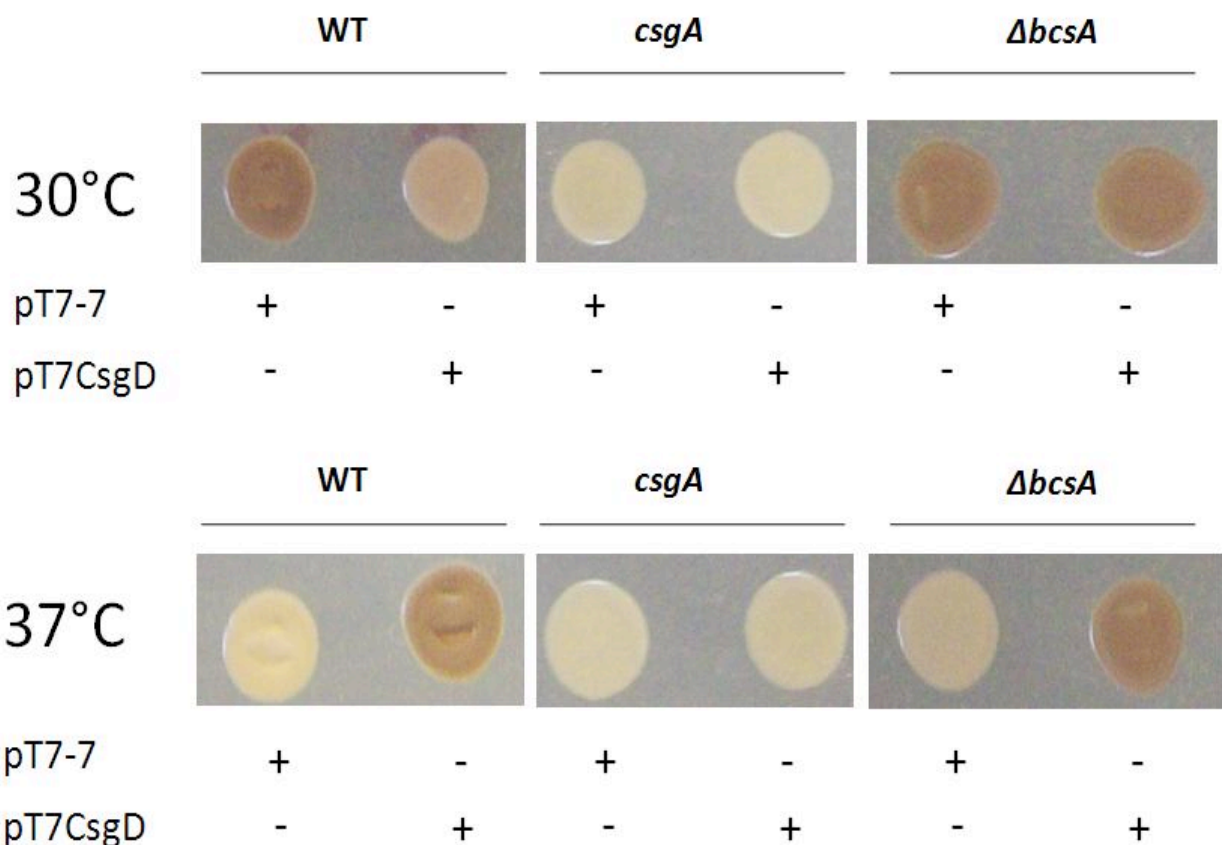
Figure 3. Phenotypes of strains MG1655 (WT), PHL856 (*csgA*) and LG26 (Δ*bcsA*) on Congo Red-supplemented agar medium (CR medium), either at 30 °C (upper panel) or at 37 °C (lower panel). Strains were transformed either with the pT7-7 control vector or with the pT7CsgD plasmid as indicated.

Thus, cellulose in our laboratory conditions doesn't seem to be an adhesion factor. In order to understand the role of cellulose in MG1655, we performed bacterial cell aggregation tests and we also tested resistance to desiccation. Several reports, indeed, point to a role for cellulose in resistance to environmental stresses such as desiccation (Gibson *et al.*, 2006). As shown in Table 2, MG1655 grown in M9Glu/sup did not form any detectable cell aggregates; however, MG1655/pT7CsgD displayed a strong aggregative phenotype at 37 °C, but not at 30 °C; deletion of *bcsA* resulted in pT7CsgD-dependent cell aggregation even at 30 °C, suggesting that cellulose plays a negative role in cell aggregation as well as in biofilm formation. In contrast, lack of *bcsA* resulted in four- to fivefold decrease in sensitivity to desiccation (Table 2). Thus, our results would suggest that in MG1655, cellulose function might be more related to resistance to environmental stresses rather than to biofilm formation and cell aggregation.

Table 2. Cell aggregation properties and resistance to desiccation.

Strain	Cell aggregation		Cell surviving exposure to dry conditions (%,±)*
	30°C	37°C	
MG1655/pT7-7	-	-	1,8 (±0,14)
MG1655/pT7CsgD	-	+ +	7,6 (±0,25)
PHL856 (*csgA*::*Kan*)/pT7-7	-	-	0,5 (± 0,07)
PHL856 (*csgA*::*Kan*)/pT7CsgD	-	-	3,5 (±0,17)
LG26 (Δ*bcsA*::*Kan*)/pT7-7	-	-	0,3 (±0,06)
LG26 (Δ*bcsA*::*Kan*)/pT7CsgD	+ +	++	1,6 (±0,11)

*Cells were incubated for 1 h at 30 °C, and values (mean of three different experiments) were determined as described in Methods.

Part II

CsgD effects on MG1655 protein production. In order to investigate the possible effects of CsgD on global gene expression in MG1655 strain, we carried out protein analysis of fractionated cell extracts on monodimensional SDS-PAGE, comparing MG1655 transformed with pT7-7 control vector and

pT7CsgD. We decided to perform a similar approach in another laboratory strain, EB1.3, which is impaired in *rpoS* expression. *rpoS* gene encodes for σ^s, an alternative sigma subunit of the RNA polymerase (Mulvey & Loewen, 1989). Unlike EB1.3, in the *rpoS*-proficient strain CsgD overexpression significantly affects protein production pattern (Fig. 4). We excised the bands differently expressed in MG1655/pT7CsgD and identified the corresponding proteins by MALDI-TOF after in-gel trypsin digestion (Table 3). We found that CsgD positively affects expression of the PflB, GadA, WrbA and Dps proteins in the cytoplasmic fraction and of Dps, CsgG and OmpW proteins in the outer membrane fraction. Interestingly, four of the six proteins produced at higher level in the presence of CsgD, namely GadA (band 2 in Fig. 4), WrbA (band 5), Dps (bands 6, 7 and 11) and CsgG (band 9), are encoded by genes known to belong to the *rpoS* regulon, i.e. their transcription depends upon σ^s factor. The GadA, WrbA and Dps proteins are, respectively, a glutamate decarboxylase involved in resistance to acid stress (De Biase *et al.*, 1999; Smith *et al.*, 1992), a quinone reductase part of a response to oxidative stress (Natalello *et al.*, 2007; Patridge and Ferry, 2006) and a bacterial ferritin able to protect DNA from iron-mediated hydroxyl-radical formation (Martinez and Kolter, 1997; Zhao *et al.*, 2002). Interestingly, two bands corresponding to the Dps protein were found in the cytoplasmic fraction of MG1655/pT7CsgD (bands 6 and 7, Fig. 4A); this would suggest the existence of different Dps isoforms, as also observed in two-dimensional gel analysis of the MG1655 proteome (Lelong *et al.*, 2007). Despite Dps being a cytoplasmic protein, we also detected its presence in the outer membrane fraction (band 11, Fig. 4C), as already report for other biofilm-forming *E. coli* strain (Lacqua et al., 2006), thus suggesting that a fraction of the Dps protein might be associated with the outer membrane. Unlike Dps, GsgG and OmpW are outer membrane proteins (Loferer *et al.*, 1997; Pilsl *et al.*, 1999). The CsgG is a component of the curli transport system and is encoded by a gene belonging to the *csgDEFG* operon, which also encoded CsgD (Hammar *et al.*, 1995; Loferer *et al.*, 1997).
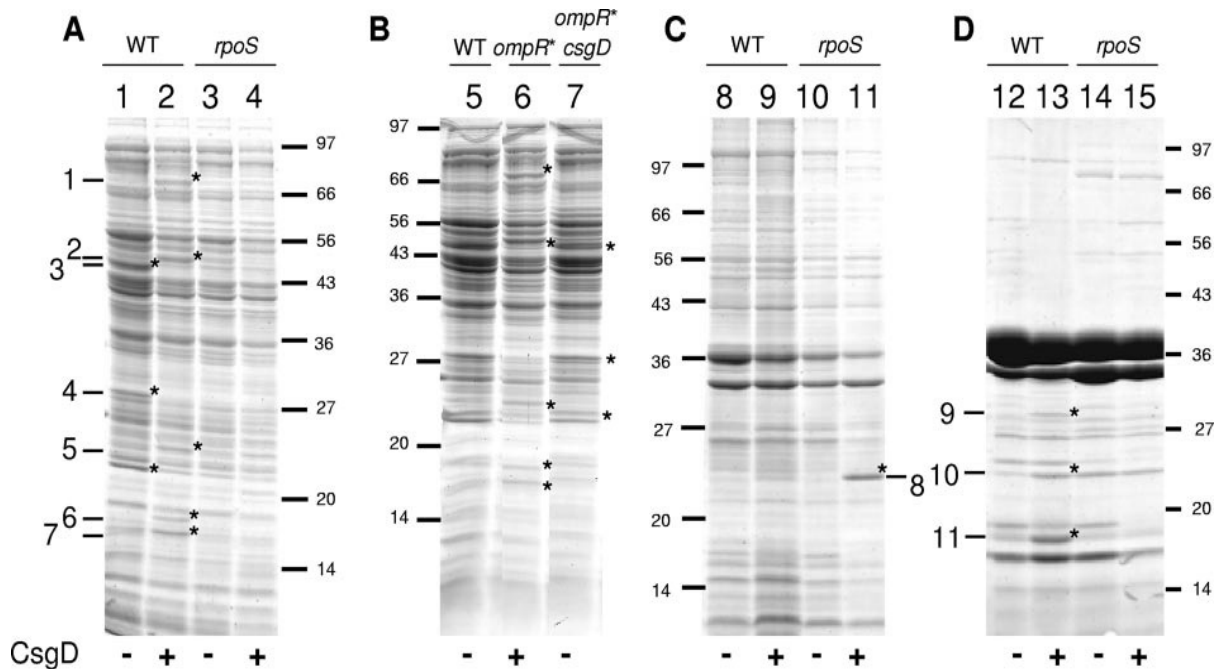
Figure 4. SDS-PAGE of fractioned cells extracts. **(A)** Cytoplasmic proteins. Lane 1, MG1655/pT7-7; lane 2, MG1655/pT7CsgD; lane 3, EB1.3/pT7-7; lane 4, EB1.3/pT7CsgD. **(B)** Cytoplasmic proteins. Lane 5, MG1655; lane 6, PHL628; lane 7, PHL1087. **(C)** Inner membrane proteins. Lane 8, MG1655/pT7-7; lane 9, MG1655/pT7CsgD; lane 10, EB1.3/pT7-7; lane 11, EB1.3/pT7CsgD. **(D)** Outer membrane proteins. Lane 12, MG1655/pT7-7; lane 13, MG1655/pT7CsgD; lane 14, EB1.3/pT7-7; lane 15, EB1.3/pT7CsgD. The relevant genotype of the different bacterial strains is indicated in the figure. *ompR** stands for the *ompR234* mutation resulting in increased CsgD expression (Vidal *et al.*, 1998). The position of molecular mass markers is shown (numbers indicate molecular masses in kilodaltons). Asterisks indicate bands differentially expressed in a CsgD-dependent manner that were excised and identified by MALDI-TOF (numbered from 1 to 11).

Consistent with their being part of the *rpoS* regulon, no CsgD-dependent increase in protein expression of GadA, WrbA, Dps and CsgG could be detected in EB1.3/pT7CsgD, the *rpoS* mutant of MG1655 transformed with the pT7CsgD plasmid (Fig. 4, lanes 4 and 11), thus suggesting that regulation by CsgD does not bypass the need for σ^s. Moreover, we found that expression of the PflB protein (band 1), encoded by a gene thus far never described as σ^s dependent, is stimulated by CsgD in the MG1655 background only, suggesting that its CsgD-dependent expression requires a functional *rpoS* gene (Fig. 4A). In contrast, expression of the OmpW protein (band 10), although stimulated by CsgD, appears to be negatively regulated by σ^s (Fig. 4, compare lane 12 and 14).

The induction of proteins belonging to the *rpoS* regulon in MG1655/pT7CsgD is strictly dependent on CsgD expression. Indeed, ectopic expression of the AdrA protein (a c-di-GMP synthase), as well as truncated

(inactive) forms of CsgD, did not lead to any detectable changes in protein expression (data not shown). In addition, the same pattern of protein regulation was observed comparing MG1655/pT7CsgD to PHL628, an *ompR234* mutant of MG1655 characterized by high level of transcription of the *csgD* gene (Prigent-Combaret *et al.*, 2001). Proteins whose production is increased in the cytoplasmic fraction of PHL628 correspond to those identified in MG1655/pT7CsgD, as determined by MALDI-TOF analysis, and their expression was totally abolished in PHL1087, a *csgD::kan* derivative of PHL628 (Fig. 4B).

Transcription activation of *rpoS*-dependent genes by CsgD. Although monodimensional SDS-PAGE can only provide an incomplete view of CsgD-mediated effects on global protein production in MG1655, these results (Fig. 4 and Table 3) suggest that CsgD might somehow affect the expression of the *rpoS* regulon. In order to confirm this possibility, we determined CsgD effects on transcription of the *dps*, *pflB* and *osmB* genes by real-time PCR, both in the MG1655 strain and its derivative EB1.3. These genes were chosen as representatives of known *rpoS*-dependent genes encoding genes whose production is stimulated by CsgD (*dps*), genes encoding proteins stimulated by CsgD but not assigned to the *rpoS* regulon (pflB), and *rpoS*-dependent genes encoding proteins for which no stimulation by CsgD could be detected in our SDS-PAGE experiments (*osmB*).

Table 3. Gene characteristics

Band N°[a]	Protein	Predicted molecular mass (Da)[c]	Gene function (reference)	Regulation of corresponding gene (reference)
1	PflB*	85,357	Pyruvate formate lyase I	Induced by ArcA and FNR
2	GadA*	52,685	Glutamate decarboxylase	Positively regulated by *rpoS*, CRP, *gadE*, *gadW*, *gadX*; negatively by HN-S
3	TnaA†	52,773	Tryptophan deaminase	Positively regulated by rpoS and CRP
3	GatZ†	47,109	Subunit of tagatose-1,6-bisphosphate aldolase 2	Positively activated by CRP; repressed by ArcA
4	GatY†	30,812	Subunit of tagatose-1,6-bisphosphate aldolase 2	Same transcription unit as *gatZ*
5	WrbA*	20,846	Quinone reductase; response to oxidative stress	Positively regulated by *rpoS*
6,7 and 11	Dps*	18,695	Bacterial ferritin	Positively regulated by *rpoS*
8	CsgD*	24,935	Transcritpional regulator	Positively regulated by *rpoS*, *hns* and *ompR*
9	CsgG*	30,557	Outer membrane protein; curli transport component	Same transcription unit as *csgD*
10	OmpW*	22,928	Outer membrane protein; receptor for colicin S4	Positively regulated by FNR

[a] In Fig.4 band 3 was identified as a mixture of two proteins and thus appears twice, once for each protein.

[b] *, Increased expression in MG1655/pT7CsgD; †, decreased expression in MG1655/pT7CsgD.

[c] Predicted molecular masses were obtained from the EcoCyc database (http://www.ecocyc.org/).

As shown in Fig. 5, CsgD activates transcription of *dps*, *pflB* and *osmB* genes by a similar extent (19- to 30-fold); however, CsgD transcription activation can only occur in the MG1655 strain, and it is totally abolished in its *rpoS* derivative EB1.3. These results confirm that CsgD-mediated effects on protein expression take place at gene transcription level and suggest that CsgD can activate expression of the *rpoS* regulon, or at least of a subset of σ^s-dependent promoters. Interestingly, both the CsgD-dependent *csgB* and the *adrA* promoters, as well as the *csgD* promoter itself, have been proposed to be under the control of σ^s and regulated by the Crl protein, a specific modulator of σ^s activity (Bougdour *et al.*, 2004; Pratt and Silhavy, 1998; Robbe-Saule *et al.*, 2007; Romling *et al.*, 1998a). Thus, it might be possible that CsgD could act as a specific activator for the σ^s-associated form of RNA polymerase (Eσ^s). In order to better understand the interplay between σ^s and CsgD, we compared the effect of the *rpoS* inactivation on CsgD transcription at either the *csgB* or the *csgD* promoter. Activation of its own promoter by CsgD is suggested by increased production of the CsgG protein (Fig. 4), encoded by a gene which is part of the *csgDEFG* operon.
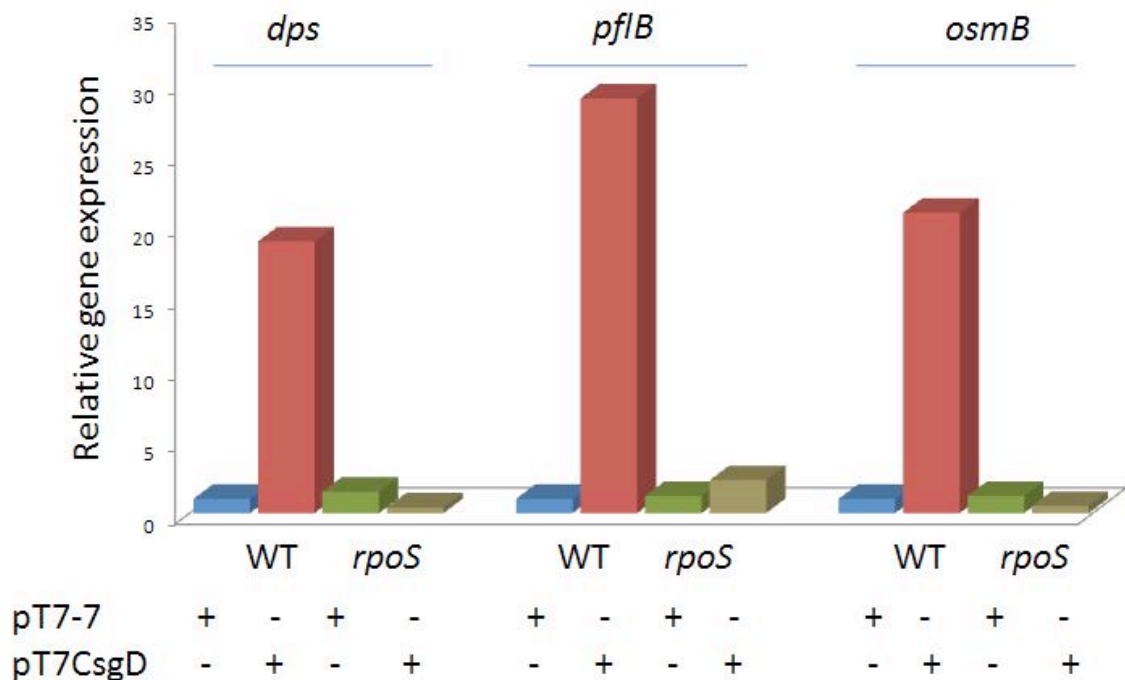
Figure 5. Relative transcription of the *dps, pflB* and *osmB* genes in MG1655 and *rpoS,* as determined by real-time PCR. Relative transcription values were set to 1 for both MG1655/pT7-7 and *rpoS*/pT7-7 for better comparison of the CsgD-dependent effects in either strain.

Thus, we transformed with either pT7-7 or pT7CsgD vector two derivatives of the MG1655 strain carrying, respectively, a *csgA::uidA* (transcription of the *csgA* gene is directed by *csgB* promoter) or a *csgD::uidA* chromosomal transcriptional fusion. Results shown in Fig. 6 indicate that CsgD expression results in increased transcription levels for both the *csgB* and *csgD* promoters. However, CsgD-dependent transcription activation at the *csgB* and *csgD* promoters greatly differs both in timing and in the extent of dependence on the *rpoS* gene: transcription from the *csgB* promoter (Fig. 6A) is activated by CsgD regardless of growth phase, and *rpoS* inactivation only results in a slight reduction (down to ca. 65%) in promoter activity. Thus, although σ^s is required for optimal transcription levels, the *csgB* promoter is activated by CsgD in a manner largely independent of σ^s, strongly suggesting that σ^s is not directly involved in protein-protein interaction between CsgD and RNA polymerase leading to transcription activation. In contrast to *csgB*, CsgD-mediated stimulation of its own promoter only takes place in late-log and stationary phase and is totally abolished in an *rpoS* strain.
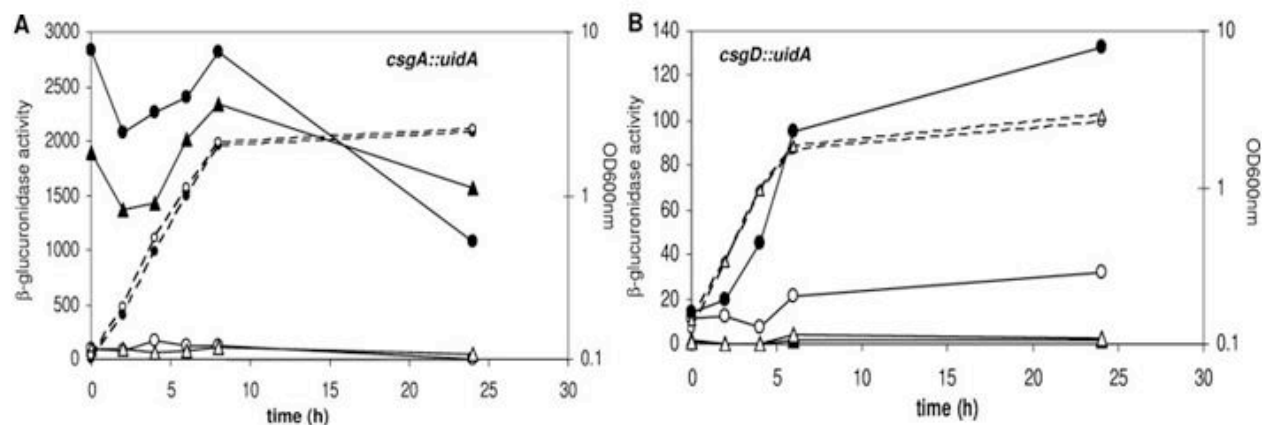
Figure 6. ß-Glucoronidase activity measured from either *csgA::uidA* (A) or from *csgD::uidA* (B) chromosomal fusions. Cultures were grown in M9Glu/sup at 30°C. **(A)** Reporter gene expression from the *csgB* promoter in PHL856 (MG1655 *csgA::uidA*, circles) and LG05 (MG1655 *rpoS csgA::uidA*, triangles) transformed either with pT7-7 (open symbols) or pT7CsgD (closed symbols). Dashed lines indicate growth curves of MG1655/pT7-7 (o) and pT7CsgD (●). **(B)** Reporter gene expression from the *csgD* promoter was measured either in PHL1088 (MG1655 *csgD::uidA*, circles) and LG07 (MG1655 *rpoS csgD::uidA*, triangles) transformed either with pT7-7 (open symbols) or pT7CsgD (closed symbols). Dashed lines indicate growth curves of MG1655/pT7-7 (o) and EB1.3/pT7-7 (●)

Effects of CsgD on σ^s intracellular concentrations. Results shown in Fig. 5, indicating that CsgD does not function as an Eσ^s-specific transcription activator, and the observation that *rpoS*-dependent promoters stimulated by CsgD (Fig. 6) lack a putative CsgD binding site would suggest that the CsgD protein might affect expression of the *rpoS* regulon by altering either σ^s activity or its intracellular concentration. In a previous work it was shown that CsgD can activate the *yaiB* gene (Brombacher *et al.*, 2006). The product of the *yaiB* (now *iraP*) gene has been shown to be a stabilization factor for the σ^s protein, which acts by inhibiting RssB-mediated degradation of σ^s in response to phosphate starvation (Bougdour *et al.*, 2006). Thus, CsgD activation of the *iraP* gene should results in improved σ^s stability and possibly increase in σ^s intracellular concentrations, resulting in increased transcription of σ^s-dependent genes. In order to confirm these results, we compared *iraP* expression in both MG1655/pT7-7 and the MG1655/pT7CsgD strain by real-time PCR experiments. As shown in Fig. 7, CsgD increases *iraP* transcription levels of ~ 15-fold. Stimulation of *iraP* transcription of ~ 6-fold was also observed when we compared MG1655 to its *ompR234* mutant derivative PHL628 (Fig. 7), which expresses the *csgDEFG* operon at higher levels than MG1655 (Prigent-Combaret *et al.*, 2001). Activation of *iraP* transcription in the *ompR234* mutant strain was totally abolished by *csgD* inactivation (Fig. 7), thus confirming that *iraP* activation observed in PHL628 depends on CsgD. CsgD-induced *iraP* expression in either MG1655/pT7csgD or PHL628 is consistent with increased production of proteins encoded by *rpoS*-dependent genes observed in both strain (Fig. 4).
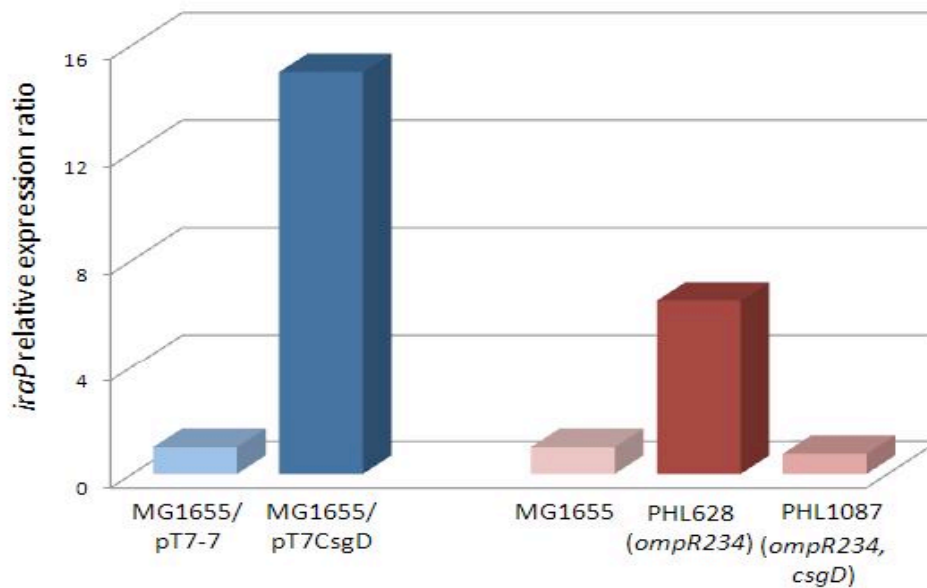
Figure 7. Expression levels of the *iraP* gene, normalized to 16S rRNA, are shown as relative values in MG1655/pT7CsgD in comparison to MG1655/pT7-7, as well as in PHL628 and PHL1087 in comparison to MG1655. Samples were taken from cultures grown overnight in M9Glu/sup at 30 °C. Values shown are the average of three independent experiments, with a standard deviation always lower than 10%.

In order to verify that CsgD expression might stimulate the *rpoS* regulon by IraP-dependent stabilization of σ^s, we compared the levels of σ^s intracellular amount in both MG1655/pT7-7 and MG1655/pT7CsgD by Western blotting experiments (Fig. 8A). Expression of CsgD from the pT7CsgD plasmid resulted in a clearly detectable increase in σ^s intracellular amounts in MG1655 strain. As expected, no bands reacting with anti- σ^s antibodies were detected in the *rpoS* mutant EB1.3, regardless the presence of pT7CsgD (lanes 5 and 6). Inactivation of the *iraP* gene completely abolishes CsgD-dependent increase in σ^s intracellular concentrations (lane 4), strongly suggesting that this effect indeed takes place through IraP-mediated stabilization of the σ^s protein. In the growth conditions we tested, σ^s intracellular levels do not differ significantly in MG1655 compared to its *iraP* mutant derivative (Fig. 8A, lane 1 and 3), a finding in agreement with previous results that indicate that IraP is only essential for σ^s stability under phosphate starvation conditions (Bougdour *et al.*, 2006). In order to correlate σ^s intracellular concentration and expression of σ^s-dependent genes, cell extracts from the MG1655, EB1.3 (*rpoS*) and LG03 (*iraP*) strains were compared by SDS-PAGE. As shown in Fig. 8B, a strict correlation exists between σ^s intracellular concentration and expression of proteins encoded

by σ^s-dependent genes. Consistent with the lack of CsgD-dependent increase in σ^s intracellular concentration in LG03 (*iraP*; Fig. 8A, lane 4), the CsgD protein failed to stimulate expression of the GadA, WrbA and Dps proteins in the strain (Fig. 8B, lane 4). The band running with electrophoretic mobility similar to WrbA in cell extracts of EB1.3 and LG03 was identified as adenosine phosphoribosyltransferase and its expression appears to be CsgD independent (Fig. 8B). To confirm that CsgD-dependent accumulation of σ^s is mediated by its stabilization via the IraP protein, we performed σ^s protein turnover assays in cell extracts of either MG1655 or its *iraP* mutant derivative (LG03), grown both in the presence or absence of the pT7CsgD plasmid. As shown in Fig. 9, the stability of the σ^s protein is increased in cell extracts of MG1655/pT7CsgD compared to MG1655/pT7-7; however, no CsgD-dependent σ^s stabilization could be detected in cell extracts of the LG03/pT7CsgD strain, consistent with a direct role of the IraP protein in CsgD-mediated σ^s stabilization. In the absence of CsgD expression, the σ^s half-life in cell extracts of the *iraP* mutant is similar to MG1655 (14 versus 12 min, respectively), as detected by image analysis.
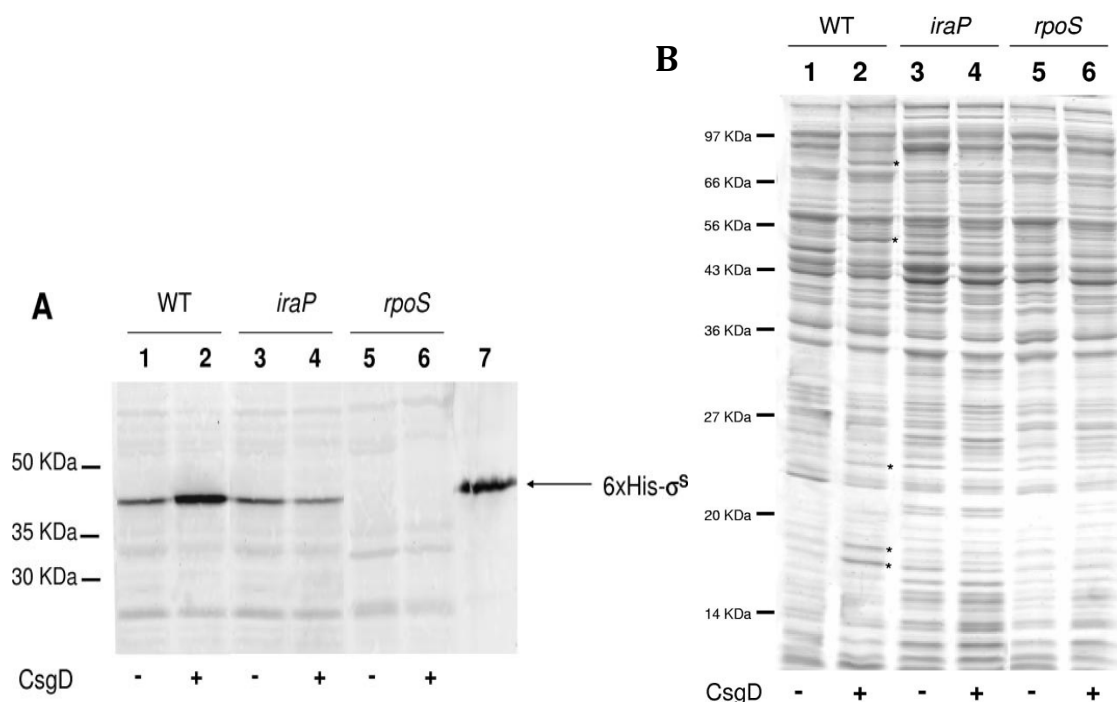
Figure 8. (A) Western blotting with anti-σ^s antibodies. Cell extracts were prepared from overnight cultures grown in M9Glu/sup at 30 °C and 20 μg of total proteins were loaded onto a SDS-polyacrylamide gel. **(B)** SDS-PAGE analysis of cytoplasmic proteins. Differently expressed proteins are indicated by an asterisk.
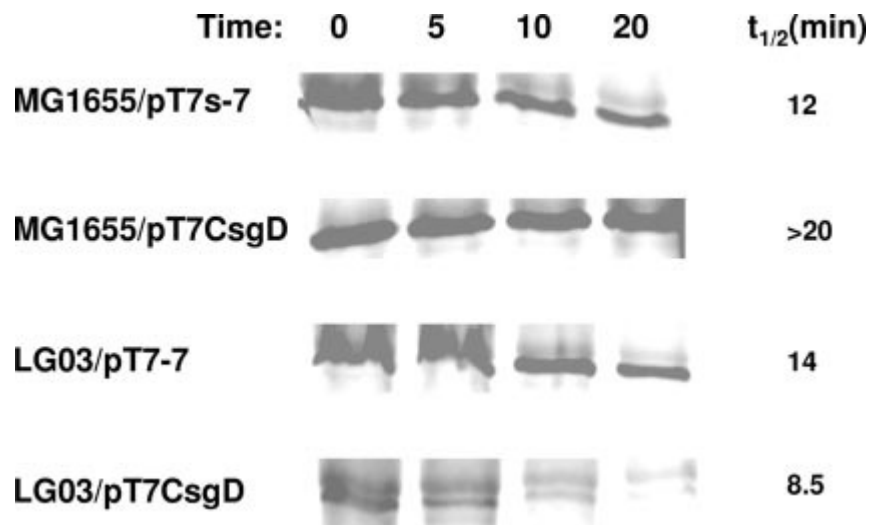
Figure 9. σ^s turnover experiments. Cell extracts of MG1655/pT7-7, MG1655/pT7CsgD, LG03/pT7-7 and LG03/pT7CsgD were incubated at 37 °C. Samples (50 µg of proteins) were taken at the indicated times and loaded onto a SDS-polyacrilamide gel. The amount of σ^s protein was determined by Western blotting. σ^s half-life values were obtained by quantification of the σ^s-corresponding bands using the ImageQuant 5.2 image analysis program.

DISCUSSION

In this work, we analyzed the effects of CsgD overexpression on biofilm formation and σ^s stabilization. The CsgD protein activates transcription of the *csgBA* operon and the *adrA* gene, thus leading to production of two extracellular structures: curli fibers and cellulose. Co-ordinated production of both curli and cellulose was initially proposed to be functional in cell adhesion and biofilm formation (Romling *et al.*, 2000). However, more recent report pointed to a negative role for cellulose in curli-mediated interaction (Wang *et al.*, 2006). Our results strongly suggest that cellulose overproduction in a *csgD*-overexpressing derivative of the MG1655 laboratory strain of *E. coli* negatively affects biofilm formation (Fig. 1) and cell aggregation (Table 2). These results indicate that cellulose might act as a weak adhesion factor in strain poorly efficient in curli production, but its production is detrimental for biofilm formation in bacterial

strains that strongly produce curli (Fig. 1), thus suggesting that cellulose modulates cell's adhesion properties. Since cellulose production does not inhibit curli-dependent biofilm formation through negative regulation of curli-encoding genes, a possible mechanism for inhibition of curli-mediated adhesion by cellulose might be physical masking of curli fibres by excessive cellulose production, similar to the effect of other capsular polysaccharides on proteinaceous adhesion factors (Schembri *et al.*, 2003). In contrast to the effect on biofilm formation, cellulose appears to play a role in protection against environmental stresses such as desiccation (Table 2), in agreement with previous observations in *Salmonella spp* (White *et al.*, 2006). Interestingly, even *csgA* inactivation results in a decreased resistance to desiccation (Table 2), suggesting that the curli/cellulose matrix might confer better protection than cellulose alone.

Beside its role in curli and cellulose activation, the CsgD protein can activate the expression of the *iraP* gene (Bougdour *et al.*, 2006). The IraP protein acts as a stabilization factor for σ^s factor by binding to the RssB protein and thus preventing σ^s proteolysis by the RssB-ClpXP protein complex. IraP-dependent stabilization of σ^s takes place in response to phosphate starvation. In this work, we showed that CsgD transcription activation of the *iraP* gene does indeed result in a significant increase on σ^s intracellular concentration by positively affecting σ^s protein stability (Fig. 8 and 9), with altered expression of σ^s-dependent genes (Table 1, Fig. 4, 5, 8B). Our experiments were performed in phosphate-rich medium, suggesting that IraP-mediated σ^s stabilization might take place in response to environmental signals other than phosphate starvation; in the *csgD*-expressing PHL628 strain of *E. coli*, CsgD-dependent *iraP* transcription can only take place in response to environmental signals leading to CsgD expression and curli production, i.e., low temperature and osmolarity (stress conditions).
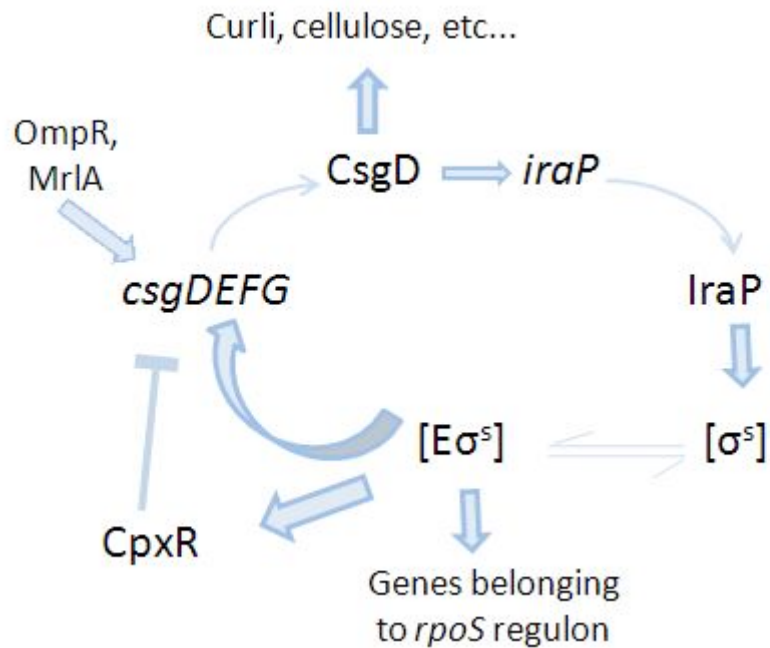
Figure 10. Model of the CsgD/σ^s autoactivation loop. Thin light blue arrows indicate gene expression; block light blue arrows indicate positive regulatory interactions; CpxR blocks *csgDEFG* expression. See the text for details.

CsgD-mediated increase of σ^s cellular concentrations via the *iraP* gene would trigger an autoactivation loop, as summarized in Fig. 10: an increase in the cellular σ^s pool in conditions permitting *csg* expression (*i.e.*, low osmolarity) leads to increased transcription from the *csgD* promoter; in turn, production of the CsgD protein results in *iraP* activation and consequent further stabilization of σ^s. Evidence for this mechanism in the MG1655 strain is provided by σ^s-dependent activation of the *csgD* promoter in the presence of pT7CsgD (Fig. 6B) and consequent production of proteins encoded by the *csgDEFG* operon, such as CsgG (Fig. 4C), in response to CsgD-dependent accumulation of σ^s. A possible feedback control for this regulation loop could be provided by the CpxA/CpxR two-component regulatory system, whose activity is triggered by curli overexpression and results in repression of both *csgD* and *csgB* promoters (Prigent-Combaret *et al.*, 2001). Consistent with this model, transcription of the *cpxRA* operon is itself positively controlled by σ^s (De Wulf *et al.*, 1999). A more general role of CsgD- σ^s interaction would be to coordinate the production of adhesion and cell aggregation factors, relaying the transition from single cell to biofilm to expression of the *rpoS* regulon, i.e one of the main stress responses in bacteria (Hengge-Airons, 2002).

METHODS

Bacterial strains and growth conditions. All strains and plasmids used are listed in Table 3. Bacteria were grown in M9Glu/sup medium (M9 minimal medium supplemented with 0.5% glucose and 2.5% Luria broth) at 30 °C or 37 °C. When needed, antibiotics were used at the following concentrations: ampicillin, 100 µg/ml; tetracycline, 25 µg/ml; kanamycin, 50 µg/ml and chloramphenicol, 35 µg/ml. For growth on Congo-red-supplemented agar medium, bacteria were inoculated in M9Glu/sup medium in a microtitre plate and then spotted using a replicator, on Congo red medium (1% Casamino acids, 0.15% yeast extract, 0,005% $MgSO_4$, 2% agar) to which 0,004% Congo red and 0,001% Comassie blue were added after autoclaving. Both dyes were dissolved in 50% ethanol to a final concentration of 0.2%.

Biofilm formation assays. Biofilm formation in microtiter plates was determined essentially as described previously (O'Toole and Kolter, 1998). Cells were grown in liquid cultures in microtiter plates (0.2 ml) for 18 h in M9Glu/sup at 30 °C or at 37 °C. The liquid culture was removed, and the cell optical density at 600 nm (OD_{600}) was determined spectrophotometrically. Cells attached to the microtiter plates were washed with 0.1 M phosphate buffer (pH 7.0) and then stained for 20 min with 1% crystal violet (CV). The stained biofilms were thoroughly washed with water and dried. CV staining was visually assessed, and the microtiter plates were scanned. For semiquantitative determination of biofilms, CV-stained cells were resuspended in 0.2 ml of 70% ethanol, and their absorbance was measured at 600 nm and normalized to the OD_{600} of the corresponding liquid culture. To determine cell aggregation, 2 ml cultures were grown overnight in 15 ml Falcon tubes with vigorous shaking, then left standing at room temperature for 1h. Cell aggregation was determined by visual estimation of the cell sediment at the bottom of the Falcon tube.

Extracellular polysaccharide determination. Cellulose in the growth medium was determined by measuring the glucose reducing units produced after treatment with cellulase. Cultures (50 ml) were grown overnight at either 30 °C or 37 °C. Cells were pelleted by centrifugation, and the culture supernatants were lyophilized. Dried culture supernatants were dissolved in water at 100 mg/ml final concentration, and the resuspended culture supernatants were incubated with cellulase from *Trichoderma reesei* ATCC 26921 (5 mg/ml, 30 U/ml; Sigma) in sodium acetate buffer (pH 5.0) at 37 °C for 16h. Glucose released from cellulose by cellulase digestion was estimated with the procedure described by Somogyi (1952) for the determination of reducing sugar units. As a standard for cellulose quantitative determination, we used a

carboxymethylcellulose solution (5 mg/ml) in sodium acetate buffer. For both culture supernatants and carboxymethylcellulose solution three different volumes (5, 15 and 30 µl) were incubated with cellulase. The amounts of sugars released by carboxymethylcellulose degradation were used as calibration curve. For culture supernatants, the amounts of reducing sugars were expressed at the percentage of total dry mass after lyophilization. In culture supernatants of the *bcs* mutant LG26 strain, used as negative control, the percentage of glucose released by cellulase treatment never exceed 0.05%.

Gene expression determination by real-time PCR. RNA isolation, cDNA synthesis, and real time- PCR analysis. For RNA isolation, strains were grown in M9Glu/sup at 30 °C to stationary phase (OD_{600} ~2). The cells were harvested by centrifugation at 13,000 rpm for 5 min at 4°C, and total RNA was extracted by using an RNeasy minikit (QIAGEN). RNA samples were checked by agarose gel electrophoresis to assess the lack of degradation and then quantified spectrophotometrically. Genomic DNA was removed by DNase I treatment. Reverse transcription was performed on 1 µg of total RNA, along with negative control samples incubated without reverse transcriptase. cDNA synthesis efficiency was verified by electrophoresis on agarose gel in comparison to negative controls. Real-time PCR was performed by using the SYBR green PCR master mixture, and the results were determined with an iCycle iQ realtime detection system (Bio-Rad). Reaction mixtures (25 µl) included 0.1 µg of cDNA and 300 nM concentrations of primers in the reaction buffer and enzyme supplied by the manufacturer. The sequences of the primers used are listed (Table 4). All reactions were performed in triplicate, including negative control samples, which never showed significant threshold cycles (C_t). The relative amounts of the transcripts were determined by using 16S rRNA as the reference gene: $C_{t(\text{gene of interest})}$- $C_{t(16s)}$=ΔC_t value.

Protein localization experiments. Cell fractionation was performed as described previously (Deflaun *et al.*, 1994). Portions (250 ml) of cultures grown in M9Glu/sup at 30 °C for 18 h were centrifuged at 4,000 X *g* for 10 min at 4 °C and washed with 5 ml of 0.1 M phosphate buffer pH 7.0 (PB). Cells were resuspended in 2 ml of PB with addition of 100 µg of lysozyme/ml and 1 mM EDTA (pH 8.0) and incubated at room temperature for 10 min. Cells were disintegrated by using a French press and centrifuged as described above to remove unbroken cells. The low-speed centrifugation supernatant was then centrifuged at 100,000 X *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 phosphatebuffered saline, left for 20 min at room temperature, and centrifuged at 40,000 X *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 0.5 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 (Chen *et al.*, 2000; performed by CRIBI, University of Padua, Padua,Italy [http://www.bio.unipd.it/cribi/]).

σ^s determination by Western blotting. Protein amounts in cytoplasmic samples were determined by the Bradford method, and 20 μg portions of total proteins were loaded onto an SDS-PAGE gel (12% acrylamide). Proteins were transferred on Hybond P membranes (Amersham Life Sciences) and incubated with the polyclonal rabbit antibodies against the σ^s protein (Robbe-Saule *et al.*, 2007). The anti-σ^s antibodies were detected by using a secondary anti-rabbit antibody conjugated with fluorescein. For σ^s turnover experiments, 50 μg of cell extracts (cytoplasmic fractions) was incubated at 37 °C for different times (0, 5, 10, and 20 min). Reactions were stopped by the addition of an equal amount of SDS-PAGE loading buffer, and the samples were used for Western blotting. Bands were quantified by using the ImageQuant 5.2 software (Molecular Dynamics).

Other methods. ß-Glucuronidase assays were performed as described previously. Bacteriophage P1vir transductions were carried out as described previously (Miller, 1972). The correctness of transduction was checked by PCR verification of the presence of the antibiotic cassette used for selection in the gene of interest, except for the *rpoS* mutants, which were checked by catalase activity assays as previously described (Visick and Clarke, 1997).

Bacteriophage P1vir transductions were carried out as described by Miller (1972). For overexpression of the AdrA protein, the *adrA* gene was amplified by PCR and the PCR product was directly cloned into the pTOPO vector. The correct orientation of the *adrA* insertion (i.e. under the control of the *lac* promoter) was confirmed by digestion with EcoRI/EcoRV, which gives two distinct digestion patterns depending on orientation of the *adrA* gene.

Desiccation experiments were performed as follows. Overnight cultures grown at 30 °C in M9Glu/sup were diluted 1:100 in H_2O. To determine bacterial concentration in the suspension before desiccation, 20 ml was spotted on a glass slide, to which 80 ml H_2O was added immediately. Serial dilutions (10^{-2} to 10^{-5}) were plated on LB agar. Typical c.f.u. ml^{-1} values for the bacterial suspensions used in the desiccation experiments ranged between 10^7 and 2.5×10^7. For the desiccation assay, 20 ml of the bacterial suspension was spotted on a glass slide and allowed to air-dry at 30 °C for 1 h, a time sufficient for full drying of the suspension drop. The dried suspension was resuspended in 100 ml H_2O, and serial dilutions (10^{-1} to 10^{-4}) were plated on LB agar and incubated overnight at 37 °C. The percentage of cells surviving drying was calculated as recovered cells (c.f.u. ml^{-1}) divided by the number of cells (c.f.u. ml^{-1}) spotted on the glass slide. Efficient recovery of bacterial cells from the glass slide after exposure to dryness was verified by direct microscopic observation (at 100 x magnification).

Curli subunit determination by SDS-PAGE was performed after formic acid solubilization of membrane-associated proteins. Samples (50 ml) of cultures grown in M9Glu/sup at 30 °C for 18 h were centrifuged at 4000 *g* for 10 min at 4 °C and washed with 5 ml 0.1 M sodium phosphate buffer pH 7.0 (PB). Cells were resuspended in 1 ml PB with addition of 100 mg lysozyme ml^{-1} and 1 mM EDTA pH 8.0 and incubated at room temperature for 10 min. Cells were disintegrated using a French press and centrifuged at 30.000 *g* for 30 min. The pellet was dissolved in 1 ml PB and treated with formic acid as described by Collinson *et al.* (1991).

Table 3. Strain and plasmids used in this work.

Strain and plasmid	Relevant genotype or characteristics	Reference or source
E. coli		
MG1655	*E. coli* K-12 λ⁻ F⁻, *rph-1*	Blattner *et al.*, 1997
EB1.3	MG1655 *rpoS::*Tn10	Prigent-Combaret *et al.,* (2001)
LG03	MG1655 *iraP::Kan*	This study
LG05	EB1.3 *csgA::uidA*-kan	This study
LG07	EB1.3 *csgD::uidA*-kan	This study
LG20	MG1655*crl::*920*cam*; obtained by transduction from LP468 (Pratt & Silhavy, 1998)	This study
LG26	MG1655Δ*bcsA*::*kan*; obtained by P1vir transduction from MG1655*bcsA* (gift from C. Beloin)	This study
PHL1087	PHL628 *csgD::uidA*-kan	Prigent-Combaret *et al.,* (2001)
PHL1088	MG1655 *csgD::uidA*-kan	Prigent-Combaret *et al.,* (2001)
PHL628	MG1655 *ompR234*	Prigent-Combaret *et al.*, (2001)
PHL856	MG1655 *csgA::uidA*-kan	Prigent-Combaret *et al.*, (2001)
Plasmid		
pT7-7	Control vector, ampicillin resistance, T7 RNA polymerase-dependent promoter	S. Tabor, Institute of Cancer research, UK
pT7CsgD	*csgD* gene cloned into plasmid pT7-7 as a 651 bp *NdeI/PstI* fragment	Prigent-Combaret *et al.*, (2001)
pTOPO	Control vector allowing direct cloning of PCR, products ampicillin kanamycin resistance	Invitrogen
pTOPOAdrA	*adrA* gene cloned as PCR product into pTOPO vector	This study

Table 4. Primers used in this work.

Primers	Sequence
csgB_fw	CATAATTGGTCAAGCTGGGACTAA
csgB_rev	GCAACAACCGCCAAAAGTTT
adrA-rt_fw	GGCTGGGTCAGCTACCAG
adrA-rt_rev	CGTCGGTTATACACGCCCG
bcsA-rt_fw	GACGCTGGTGGCGCTG
bcsA-rt_rev	GGGCCGCGAGATCACC
adrA_fw	GCTCCGTCTCTATAATTTGGG
adrA_rev	ATCCTGATGACTTTCGCCGG
rpoS_fw	GGCCTTAGTAGAACAGGAACC
rpoS_rev	CCAAGGTAAAGCTGAGTCGC
iraP_fw	TGTGTGCGCAGGTAGAAGC
iraP_rev	GCGCCCCCTCTACCTGA
dps_fw	GGATGGCTTCCGCACCG
dps_rev	CCTGTCAGGAAGCCGC
pflB_fw	ACGGCTACGACATCTCTGG
pflB_rev	ACCGAAGGACATTGCAGCAC
osmB_fw	GACCGCGGCTGTTCTGG
osmB_rev	CCTAATGGCCCTGCACCC

Yddv-dos OPERON AND REGULATION OF CURLI PRODUCTION

INTRODUCTION

The experiments described in this chapter have been published in the following publication:

- **Tagliabue, L., Maciąg, A., Antoniani, D., 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(3)**:477-84.

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 isoenzymes: diguanylate cyclases (DGCs, c-di-GMP biosynthetic enzymes), and c-di-GMP phosphodiesterases (c-PDEs, with PDE activity resides in EAL or HD-GYP domains), which degrade c-di-GMP (Chang *et al.*, 2001; Tamayo *et al.*, 2005; Cotter & Stibitz, 2007). Active DGCs are characterized by an intact GGDEF motif that represents the active centre of the enzyme (the A-site) and usually also carry an I-site, i.e. a secondary and inhibitory binding site for c-di-GMP (Christen *et al.*, 2005; Malone *et al.*, 2007). Similarly, in c-PDEs, the catalytic EAL domain retains a number of functionally important, and thus highly conserved, amino acids (Rao *et al.*, 2008; Schmidt *et al.*, 2005). c-di-GMP promotes biofilm formation in Gram negative bacteria by stimulating the production of adhesion factors by acting on c-di-GMP-responsive proteins (reviewed in Hengge, 2009), while repressing cell motility (Kader *et al.*, 2006; Mèndez-Ortiz *et al.*, 2006; Weber *et al.*, 2006; Jonas *et al.*, 2008). In addition, c-di-GMP can directly affect virulence factor production in pathogenic bacteria (Kulasakara *et al.*, 2006; Hammer & Bassler, 2009). In *E. coli* and other enterobacteria, the production of curli fibers and cellulose, which form an extracellular matrix promoting biofilm formation (Römling *et al.*, 1998a; Prigent- Combaret *et al.*, 2000; Gualdi *et al.*, 2008), is strongly stimulated by c-di-GMP (Zogaj *et al.*, 2001; Kader *et al.*, 2006; Weber *et al.*, 2006). At least six different genes encoding c-di-GMP-related proteins are

involved in curli gene regulation (Sommerfeldt *et al.*, 2009). The expression of several DGC-encoding genes, as well as curli-encoding genes, is controlled by σ^S, an alternative s factor mainly active under slow growth conditions and in response to cellular stresses. The *yddV-dos* operon is the most expressed among σ^S-dependent genes encoding enzymes related to c-di-GMP metabolism (Weber *et al.*, 2006; Sommerfeldt *et al.*, 2009). The *yddV-dos* operon encodes, respectively, a protein with DGC activity and a c-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 (Mèndez-Ortiz *et al.*, 2006), the targets of *yddV*-dependent biofilm induction have not yet been identified. In this work, we have investigated the role of the *yddV-dos* operon in the regulation of curli production.

RESULTS

Partial deletion of the *yddV* and *dos* genes. We investigated the possibility that the *yddV-dos* operon might affect the production of curli fibers by constructing two mutants in either the *yddV* or the *dos* genes. 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 heme-binding and sensor domains. Because *yddV* and *dos* are part of the same transcriptional unit (Mèndez- 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 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.

Effects of the *yddV* and *dos* mutations on Congo red binding and biofilm formation. In order to determine the possible effects of mutations in *yddV* DGC domain on curli production, we performed Congo red-binding assays using CR medium. Curli fibers bind CR with very high affinity, due to their ß-amyloid fibers. Congo red can bind, albeit with a lower affinity, other cell surface-exposed structures, such as the extracellular polysaccharides 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 (Fig. 1a). 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). In addition, we constructed plasmids carrying mutant alleles of either gene (pGEM-YddV$_{GGAAF}$ and pGEMDos$_{AAA}$, Table 3), in which the coding sequence for the amino acids responsible for either DGC activity (in the YddV protein) or c-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; Antoniani *et al.*, 2010). 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 c-PDE activity (Kirillina *et al.*, 2004; Bassis & 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 (Fig. 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 (Fig. 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 c-PDE activity.
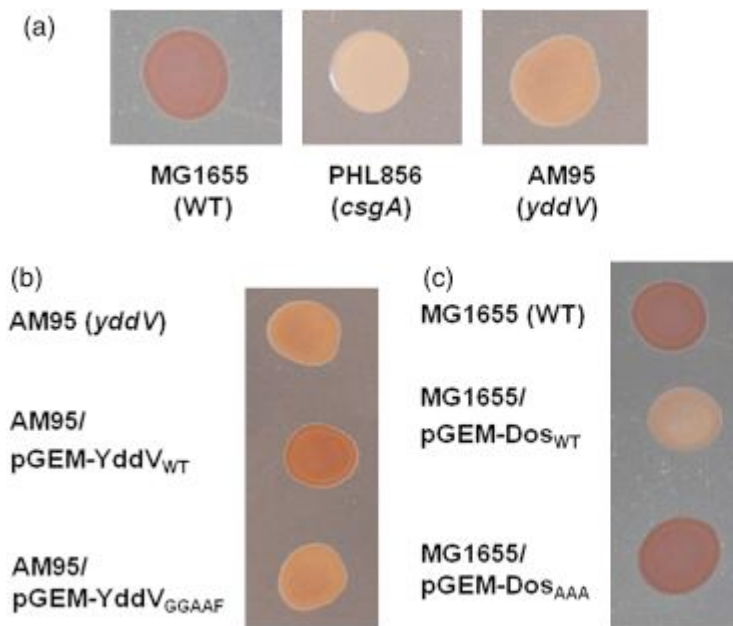
Figure 1. (a) Congo red phenotype of MG1655 (WT), PHL856 (*csgA*) and AM95 (*yddV*). **(b)** AM95 (*yddV*) strain transformed with either pGEM-YddV$_{WT}$ or pGEMYddV$_{GGAAF}$. **(c)** MG1655 strain transformed with either pGEM-Dos$_{WT}$ or pGEM-Dos$_{AAA}$.

So, being curli fibers the main determinant for adhesion to abiotic surfaces in *E. coli* MG1655 strain, we set up biofilm formation experiments on polystyrene microtitre plates in order to confirm Congo red binding results (Fig. 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 (Fig. 2), as well

as by growth at 37 °C (see Chapter II, Fig. 1), the temperature at which curli fibers are not produced in most enterobacteria (Römling *et al.*, 1998a). 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, in agreement with CR phenotypes (Fig. 1b).
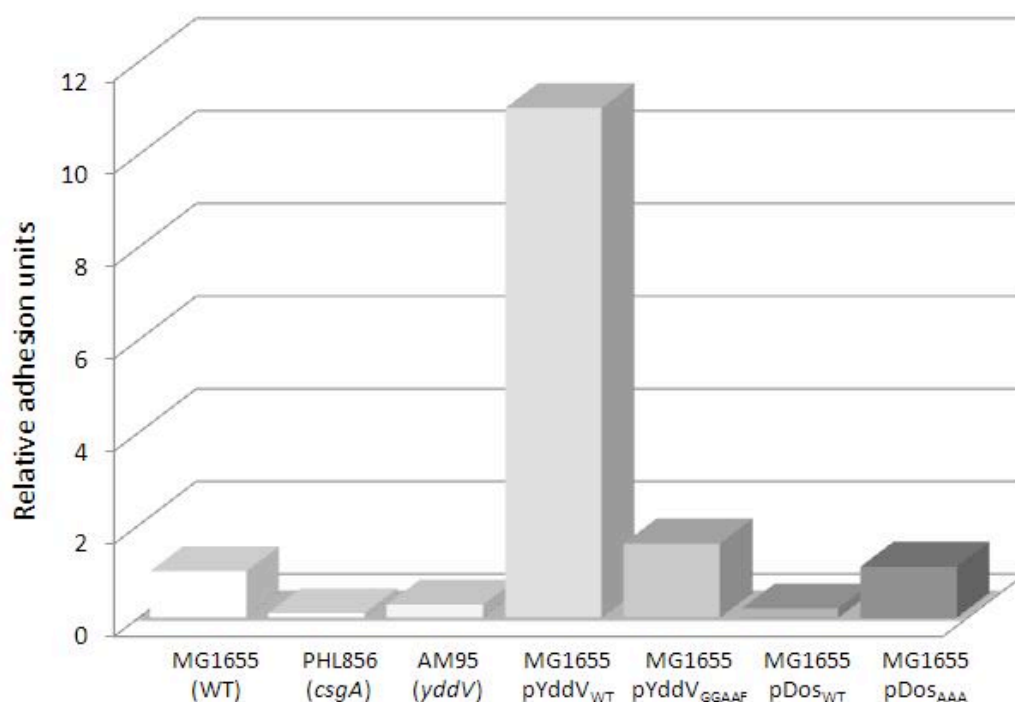
Figure 2. Surface adhesion on polystyrene microtitre plates by strains MG1655 (WT), PHL856 (*csgA*), AM95 (*yddV*), and MG1655 transformed with pGEM-YddV$_{WT}$, pGEM-YddV$_{GGAAF}$, pGEM-Dos$_{WT}$ and pGEM-Dos$_{AAA}$. The relative adhesion value was set to 1 for MG1655; the actual adhesion unit for MG1655 was 3.1. Results are the average of three independent experiments, with standard deviations always lower than 10%.

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 c-PDE activity (Fig. 2). Thus, the results of CR binding and biofilm formation strongly support the hypothesis that the YddV and Dos proteins control curli production through the modulation of intracellular c-di-GMP concentrations.

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). Because 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_{600} nm = 0.25), late exponential phase (OD_{600} nm = 0.7) and stationary phase (overnight cultures, OD_{600} nm ≥ 2.5). Transcription levels of the *csgB* and *csgD* genes in M9Glu/sup medium at 30 °C were determined by qRT-PCR (Table 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). 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 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 c-PDE activities, respectively (Table 2).

Table 1. Relative expression of *csgB* and *csgD* genes in MG1655 vs. AM95 (*yddV::cat*)

Genes	csgB		csgD		adrA	
Strains	MG1655 (WT)	AM95 (*yddV*)	MG1655 (WT)	AM95 (*yddV*)	MG1655 (WT)	AM95 (*yddV*)
Growth condition						
Early exponential (OD$_{600}$=0.25)	1*	0.7	1*	0.6	ND	ND
Late exponential (OD$_{600}$=0.7)	0.8	0.9	1.5	0.7	ND	ND
Stationary (OD$_{600}$≥2.5)	391	0.9	1.4	0.6	1*	0.74
Stationary, anoxic (OD$_{600}$≥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.

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 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 c-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$_{\Delta931-1383}$::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 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 2).

Table 2. Relative expression of *csgB* and *csgD* genes in response to either YddV or Dos overexpression.

Strains	*csgB* expression (aerobic)	*csgB* expression (anoxic)	*csgD* expression (aerobic)
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.

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 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.
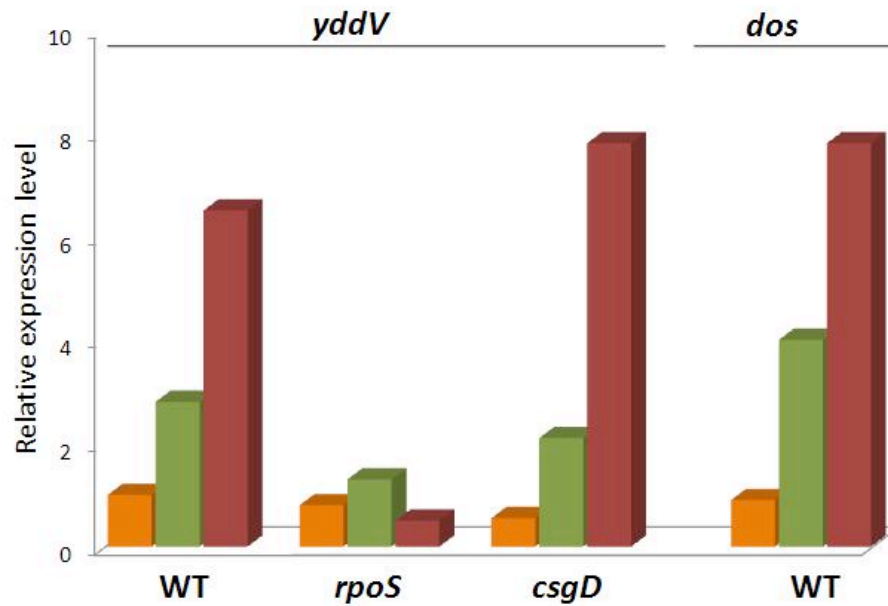
Figure 3. Relative expression levels of the *yddV* gene in strains MG1655 (WT), EB1.3 (*rpoS*) and AM75 (*csgD*), and of the *dos* gene in MG1655, as measured by realt-time PCR experiments. Expression values in MG1655 in the early exponential growth phase (OD$_{600}$nm=0.25; orange bars) (corresponding 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$_{600}$nm=0.7; green bars) and stationary phase (OD$_{600}$nm≥2.5; red bars). Data are the average of two independent experiments, each performed in duplicate.

As shown in Fig. 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 (Fig. 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.

# DISCUSSION

Curli fibers are a major adhesion factor in *E. coli* and their production is subjected to multiple forms of regulation. In this work we showed that even the *yddV-dos* operon, encoding a YddV-Dos protein complex involved in c-di-GMP

biosynthesis and turnover, affects curli production. Control of curli production by *yddV-dos* takes place at the level of transcription regulation of the *csgBAC* operon, encoding curli structural subunits (Table 1), and is mediated by the DGC and PDE activities of YddV and Dos (Fig. 1b and c; Table 2). In contrast, the YddV–Dos protein complex does not strongly influence *csgDEFG* expression, nor does it affect the expression of the CsgD-dependent *adrA* gene, encoding a positive effector for cellulose biosynthesis (Table 1). Regulation of the *csgBAC* operon, but not of *csgDEFG*, has already been described for another DGC, the product of the *yeaP* gene (Sommerfeldt *et al.*, 2009). Thus, in *E. coli*, the production of curli and cellulose involves DGC and c-PDE proteins at various levels (summarized in Fig. 4). Indeed, *csgDEFG* transcription is regulated by the DGC YdaM (Weber *et al.*, 2006) and by the PDEs YciR and YhjH (Pesavento *et al.*, 2008), *csgBAC* transcription by YeaP and by the YddV–Dos complex (Sommerfeldt *et al.*, 2009; this work), while the AdrA protein activates cellulose production (Zogaj *et al.*, 2001). The involvement of such a large number of c-di-GMP-related proteins might depend on the need to relay different environmental signals to the activation of the *csgD* regulon. Indeed, curli and cellulose production responds to a variety of environmental cues, including low temperature, slow growth and low osmolarity (Römling *et al.*, 1998b). In addition, devoted DGCs and PDEs can trigger the expression of individual CsgD-dependent genes (e.g. *csgBAC*) in response to specific environmental signals, thus altering the relative expression of genes belonging to the CsgD regulon and, in particular, the balance between curli and cellulose production. Depending on the prevalence of either its DGC or its c-PDE activities, the YddV–Dos complex can either activate or repress *csgBAC* expression. In the bacterial cell, this could be achieved by changing the relative intracellular concentrations of either YddV or Dos proteins, for instance through post-transcriptional regulation. However, our results seem to rule out the possible regulation of either *yddV* or *dos* at the level of mRNA stability (Fig. 3), suggesting that YddV and Dos might be regulated either in their protein stability level or through modulation of their enzymatic activities. Both DGC activity by YddV and c-PDE activity by Dos are inhibited in the absence of oxygen; however, YddV and Dos respond differently to oxygen concentrations, which can thus affect the overall balance between DGC and c-PDE activities in the YddV–Dos protein complex (Tuckerman *et al.*, 2009). Thus, oxygen availability in the bacterial cell might

function as an environmental signal for the modulation of intracellular c-di-GMP concentrations via the YddV–Dos complex. Oxygen tension is known to regulate curli production in *Salmonella* (Gerstel & Römling, 2001). It is conceivable that oxygen sensing can be important for curli expression in relation to biofilm growth: indeed, with the exception of cells in the external layers, bacteria growing in biofilms are exposed to a gradient in oxygen availability that leads to a switch to anaerobic metabolism in the innermost biofilm layers (Borriello *et al.*, 2004; Rani *et al.*, 2007).
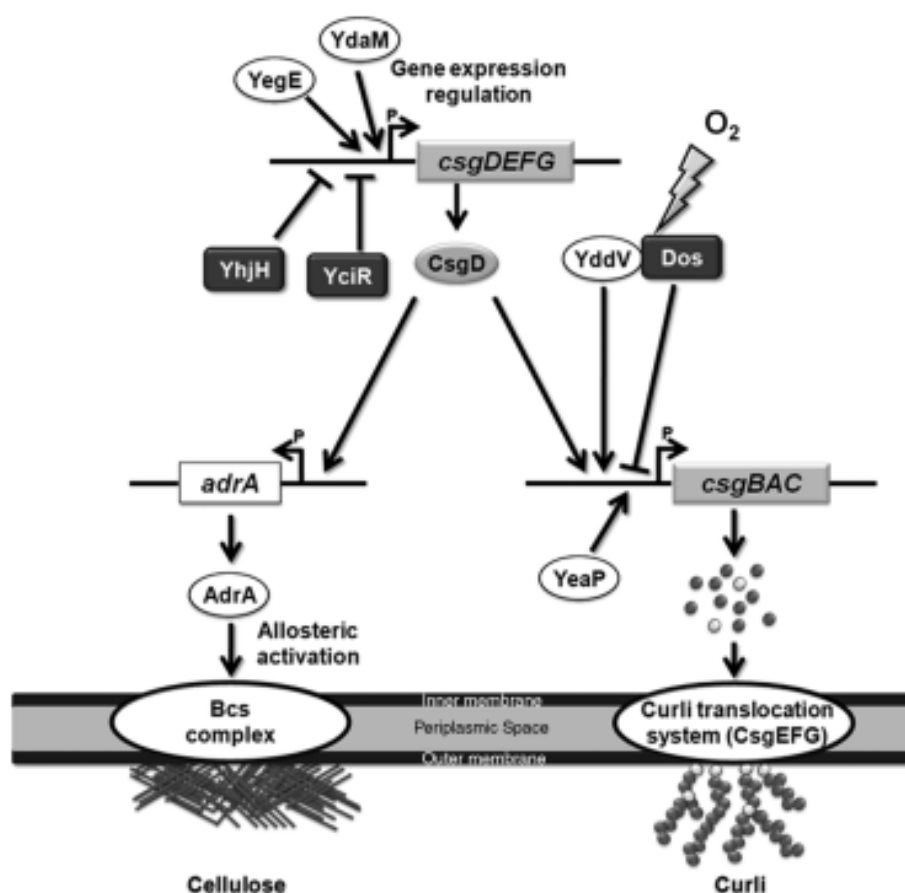
Figure 4. Model summurizing gene expression regulation of curli- and cellulose-related genes by DGC and c-PDE proteins**.** Protein with DGC activity are indicated by open ellipses; c-PDEs are shown as dark squares. The CsgD protein, which activates both the *csgBAC* operon encoding the curli structural subunit and *adrA*, encoding a DGC acting as a positive effector for cellulose biosynthesis, is indicated in the gray ellipse.

The growth of *E. coli* MG1655 in oxygen limitation results in a sevenfold decrease in *csgBAC*, but not *csgDEFG*, expression (Table 1); under these growth conditions, overexpression of neither YddV nor Dos affects *csgBAC* expression (Table 2), consistent with the inhibition of both DGC and c-PDE

activities of the YddV and Dos proteins in the absence of oxygen. This result would suggest that in the innermost biofilm layers, oxygen limitation might lead to reduced *csgBAC* expression and curli production, without, however, affecting the expression of *csgDEFG* and of other genes belonging to the CsgD regulon. We conclude that relay of oxygen sensing to curli production is mediated by c-di-GMP signalling involving the YddV–Dos complex.

METHODS

Bacterial strains and growth conditions. The bacterial strains used in this work are listed in Table 3. *E. coli* MG1655 mutant derivatives were constructed using either the λ Red technique (Datsenko & Wanner, 2000) or by bacteriophage P1 transduction (Miller, 1972). The primers used for gene inactivation and for confirmation of target gene disruption by PCR are listed in Table 4. Bacteria were grown in M9Glu/sup medium (Brombacher *et al.*, 2006), a glucose based medium supplemented with 2.5% Luria–Bertani medium as a source of amino acids and vitamins. For growth under anoxic conditions, liquid cultures were grown with no shaking in 15-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; chloramphenicol, 50 μg/ml; tetracycline, 25 μg/ml; and kanamycin, 50 μg/ml. For Congo red binding assays, bacteria grown overnight in a microtiter plate were spotted, using a replicator, on Congo red supplemented medium (CR medium), composed of 1.5% agar, 1% Casamino acids, 0.15% yeast extract, 0.005% $MgSO_4$ and 0.0005% $MnCl_2$, to which 0.004% Congo red and 0.002% Coomassie blue were added after autoclaving. Bacteria were grown for 20 h at 30 °C; phenotypes were better detectable after 24–48 h of additional incubation at 4 °C. Surface adhesion assays in polystyrene microtiter plates were performed as described (Dorel *et al.*, 1999).

Plasmid construction. The plasmids used in this work are listed in Table 3. For the construction of the pGEM-T Easy (http://www.promega.com/ tbs/tm042/tm042.pdf) plasmid derivatives, either the *yddV* or the *dos* genes were amplified by PCR from *E. coli* MG1655 genomic DNA and the PCR product was cloned into the plasmid, producing pGEM-YddV$_{WT}$ and pGEM-Dos$_{WT}$, respectively. The pGEM-YddV$_{GGAAF}$ and pGEM-Dos$_{AAA}$

plasmids were obtained by three-step PCR mutagenesis (Li & Shapiro, 1993) using the primers listed in Table 4. All constructs were verified by sequencing.

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 M9Glu/sup medium. Samples were taken in the early (OD_{600nm}=0.25) and late (OD_{600nm}=0.7) exponential phase and in the stationary phase (OD_{600nm}~2.5) for cultures grown aerobically, and in the stationary phase (OD_{600nm}~1.6) for cultures grown under anoxic conditions. RNA extraction, reverse transcription and cDNA amplification steps were performed as described (Gualdi *et al.*, 2007), using 16S RNA as the reference gene.

Table 3. *E. coli* strains and plasmids used in this work.

E.coli	Relevant phenotype	Reference or sources
Strains		
MG1655	K-12, F⁻λ⁻*rph1*	Blattner *et al.* (1997)
EB1.3	MG1655 *rpoS::tet*	Prigent-Combaret *et al*., (2001)
PHL856	MG1655 *csgA-uidA::Kan*	Gualdi *et al.,* (2008)
AM75	MG1655*csgD::cat*	This work
AM95	MG1655*yddV*Δ$_{931-1383}$*::cat*	This work
Plasmids		
pGEM-T Easy	Control vector, ampicillin resistance	Promega
pGEM-YddV$_{WT}$	*yddV* gene cloned in pGEM-T Easy vector	This work
pGEM-YddV$_{GGAAF}$	*yddV* allele carrying the mutation resulting in the GGDEF ➤ GGAAF change in the YddV DGCcatalytic site	This work
pGEM-Dos$_{WT}$	The *dos* gene cloned in pGEM-T Easy vector	This work
pGEM-Dos$_{AAA}$	*dos* allele carrying the mutation resulting in EAL ➤ AAA change in the Dos c-PDE catalytic site	This work

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Table 4. Primers used in this work.

Primers	Sequence	Utilization
yddV_for	CCAGCCTTATAAGGGTGTG	*yddV* cloning and mutant verification
yddV_rev	TTACCTCTGCATCCTGGC	*yddV* cloning
yddV$_{GGAAF}$_for	TACGGGGGCG**C**TG**C**ATTTATCATT	Construction of pGEM-YddV$_{GGAAF}$
yddV$_{GGAAF}$_rev	AATGATAAAT**G**CA**G**CGCCCCCGTA	Construction of pGEM-YddV$_{GGAAF}$
dos_for	AATCATGAAGCTAACCGATGCG	*dos* cloning
dos$_{AAA}$_for	ACGGCATCG**C**AGCC**GC**TGCTCGCT	Construction of pGEM-Dos$_{AAA}$
dos$_{AAA}$_rev	AGCGAGCA**GC**GGCT**G**CGATGCCGT	Construction of pGEM-Dos$_{AAA}$
yddV_cat_for	GGATGTACTGACGAAATTACTTAACCG CCGTTTCCTACCGTACCTGTGACGGAAGATCAC	*yddV* inactivation
yddV_cat_rev	CATCGGTTAGCTTCATGATTACCTCTGC ATCCTGGCGCATGGGCACCAATAACTGCCTTA	*yddV* inactivation
dos_tet_for	CCTGCACAATTACCTCGATGACCTGGTCGA CAAAGCCGTCCTAGACATCATTAATTCCTA	*dos* inactivation
dos_tet_rev	GTTAAATGAAAACCCGCGAGTGCGGGCGAG AGGAATTTGGAAGCTAAATCTTCTTTATCG	*dos* inactivation
csgD_cam_for	CTGTCAGGTGTGCGATCAATAAAAAAAGCG GGGTTTCATCTACCTGTGACGGAAGATCAC	*csgD* inactivation
csgD_cam_rev	AATGAATCAGGTAGCTGGCAAGCTTTTGCG TAAAGTAGCAGGGCACCAATAACTGCCTTA	*csgD* inactivation
csgD_rev	GCCATGACGAAAGGACTACACCG	Mutant verification
cat_rev	GGGCACCAATAACTGCCTTA	Mutant verification
tet_rev	GAAGCTAAATCTTCTTTATC	Mutant verification
16S_for	TGTCGTCAGCTCGTGTCGTGA	qRT-PCR
16S_rev	ATCCCCACCTTCCTCCGGT	qRT-PCR
csgB_RT_for	CATAATTGGTCAAGCTGGGACTAA	qRT-PCR
csgB_RT_rev	GCAACAACCGCCAAAAGTTT	qRT-PCR
csgD_RT_for	CCCGTACCGCGACATTG	qRT-PCR
csgD_RT_rev	ACGTTCTTGATCCTCCATGGA	qRT-PCR
dos_RT_for	CAGAGAAGCTCTGGGGATACA	qRT-PCR and mutant verification
dos_RT_rev	TTTTTCTCCAGCTGCAGCTCC	qRT-PCR
yddV_RT_for	GTTGAGCGGCAGTTGAAGAGT	qRT-PCR
yddV_RT_rev	TTTTTCAGCACCCGAAACCCC	qRT-PCR

YDDV CONTROLS PRODUCTION OF POLY-*N*-ACETYLGLUCOSAMINE

INTRODUCTION

The experiments described in this chapter have been published in the following publication:

- **Tagliabue, L., Antoniani, D., Maciąg, A., Bocci, P., Raffaelli, N. & 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.

As I already discussed in the previous chapters, transition from planktonic cells to biofilm is regulated by the second messenger, bis-(3',5')-cyclic diguanylic acid, better known as cyclic-di-GMP (c-di-GMP). Intracellular levels of c-di-GMP are regulated by two classes of isoenzymes: diguanylate cyclases (DGCs, c-di-GMP biosynthetic enzymes) and c-di-GMP phosphodiesterases (PDEs), which degrade c-di-GMP (Cotter & Stibitz, 2007). DGC- and PDE-encoding genes are present in high number in Gram negative bacteria, suggesting that c-di-GMP biosynthesis and degradation might constitute a mechanism for signal transduction involving c-di-GMP-responsive proteins interacting with specific DGCs. In this chapter, I have addressed the question of how different DGCs can affect specific cellular processes. Indeed, c-di-GMP-driven cell processes, such as cellulose production in *Salmonella* (Zogaj *et al.*, 2001), depend on specific interactions between a given DGC and one or more target proteins. To this aim, four different DGCs (AdrA, YcdT, YdaM and YddV) were overexpressed and their effect on production of extracellular structures was tested. For AdrA, YcdT and YdaM, a role in regulation of the extracellular structures curli, cellulose and PNAG have already been proposed (Weber *et al.*, 2006). In the previous chapter, I have shown that YddV can affect expression of curli-encoding genes in a manner dependent on oxygen availability.

The YddV protein is arguably one of the most expressed DGCs in *E. coli* (Sommerfeldt *et al.*, 2009), which underlines its importance. In this chapter, I

will show that overexpression of YddV leads to production of poly-*N*-acetylglucosamine (PNAG), an EPS able to promote biofilm formation, by triggering expression of *pgaABCD*, the PNAG biosynthetic operon. The ubiquitous exopolysaccharide, poly-*N*-acetyl (ß-1,6) glucosamine (PNAG) appears to play an important role in biofilm formation, immune evasion, and pathogenesis in a variety of bacterial species including: *S. aureus* (Cramton *et al.*, 1999; Kropec *et al.*, 2005; Cerca *et al.*, 2007), *Staphylococcus epidermidis* (Mack *et al.*, 2000; Vuong *et al.*, 2004; Cerca *et al.*, 2006) and *E. coli* (Wang *et al.*, 2004; Agladze *et al.*, 2005). PNAG was first described in *S. epidermidis* in which it is encoded by the intercellular adhesin (*ica*) locus (Heilmann *et al.*, 1996). In *E. coli*, PNAG is synthesized by the four proteins encoded within a homologous locus, *pgaABCD* (Wang *et al.*, 2004). It has been shown that posttranscriptional control of *pgaABCD* expression by the RNA-binding protein carbon storage regulator A (CsrA) regulation leads to the inhibition of biofilm formation (Wang *et al.*, 2005).

RESULTS

Overexpression of diguanylate cyclases (DGCs). In Enterobacteria, production of EPS such as poly-*N*-acetylglucosamine (PNAG) and cellulose (Römling *et al.*, 2000; Zogaj *et al.*, 2001; Boehm *et al.*, 2009), and of proteinaceous adhesion factors such as curli fibers (Kader *et al.*, 2006; Weber *et al.*, 2006) is regulated by DGC proteins and c-di-GMP biosynthesis. However, for several genes encoding putative DGCs, their functional role in production of adhesion factors has not been fully determined: for instance, *yddV*, arguably the most highly expressed DGC-encoding gene in *E. coli* (Sommerfeldt *et al.*, 2009), can activate transcription of the *csgBA* operon, encoding curli subunits (Tagliabue *et al.*, 2010); however, YddV overexpression can stimulate biofilm formation independently of curli production (Mendez-Ortiz *et al.*, 2006), thus suggesting that *yddV* can induce biofilm formation by acting on additional, not yet identified, targets. In order to study the effects of YddV on production of extracellular structures, we cloned the *yddV* gene into the p-GEM-T easy plasmid, which allows constitutive expression of cloned genes in the absence of

IPTG induction. We compared *yddV* with three different 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).
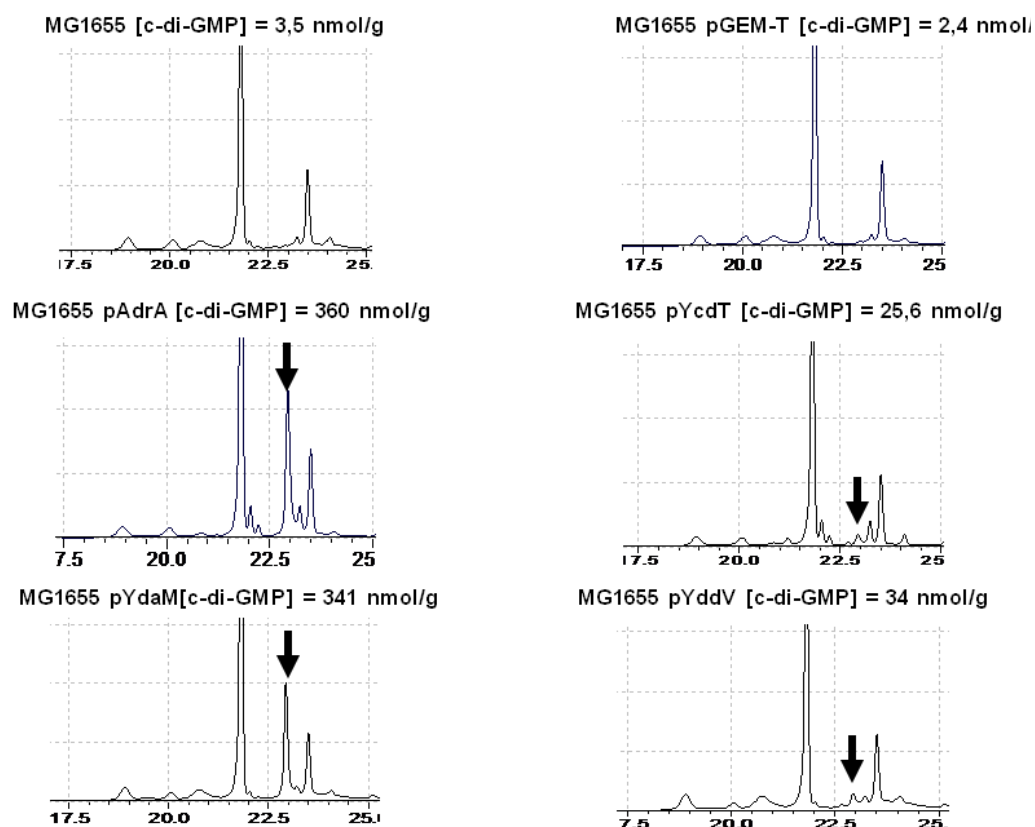
Figure 1. HPLC determination of intracellular c-di-GMP concentrations in MG1655 and in MG1655 transformed with either the pGEM-T Easy vector or pGEM-T Easy carrying the DGC-encoding genes AdrA, YcdT, YdaM and YddV. The peak corresponding to c-di-GMP is marked by an arrow; the peak with a retention time of 21.8 minutes corresponds to NAD, while the peak at 23.5 minutes was not identified.

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 (Antoniani *et al.*, 2010), YcdT and YddV only enhanced c-di-GMP concentration by ca. 10-fold (Fig. 1). 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). Expression of each DGC led to reduction in bacterial mobility (Table 1), in agreement with previous observations (Mendez-Ortiz *et al.*, 2006; Jonas *et al.*, 2008; Pesavento *et al.,* 2008).

**Table 1**. Effects of DGCs overexpression on cell motility and cell aggregation.

Bacterial strains	Cell Motility* (mm)	Aggregation**
MG1655pGEM-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 from four independent experiments.

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 (ΔcsgA::cat), unable to produce curli; LG26, a ΔbcsA::kan mutant impaired in cellulose production; AM73, a ΔcsgA/ΔbcsA double mutant, and AM56, a ΔpgaA::cat mutant unable to export PNAG and to expose it on the cell surface (Itoh *et al.*, 2008). We expected that phenotypes depending on increase in 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 bacterial cell surface to the dye Congo red (Olsen *et al.*, 1989; Zogaj *et al.*, 2001; Perry *et al.*, 1990; Gualdi *et al.*, 2008), we measured the effects of DGC overexpression on color phenotype on agar medium supplemented with Congo red (CR medium). In the absence of DGC-overexpressing plasmids, strains carrying mutations in curli-related genes (ΔcsgA and the ΔcsgA/ΔbcsA double mutant) showed a white phenotype on CR plates (Fig. 2). In contrast, inactivation of genes responsible for either cellulose (ΔbcsA) or PNAG biosynthesis (ΔpgaA) did not affect the red phenotype of the

parental strain, consistent with previous observations that in *E. coli* MG1655 Congo red-binding mostly depends on curli production (Gualdi *et al.*, 2008; Ma & Wood, 2009). 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 to the *csgA* mutant strain, but not to the Δ*csgA*/Δ*bcsA* double mutant, consistent with its role as an activator of cellulose production (Zogaj *et al.*, 2001; Antoniani *et al.*, 2010). Overexpression of YdaM did not affect CR phenotype in MG1655 and in its Δ*pgaA* mutant derivative, but it conferred a weak red phenotype on CR medium both to the curli-deficient mutant and to 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 might trigger the production of yet additional cell surface-associated structures able to bind Congo red. In contrast to AdrA and YdaM, YcdT expression led to no detectable changes in CR phenotype in any of the strains tested (Fig. 2). However, YcdT overexpression, in addition to increasing c-di-GMP intracellular concentrations (Fig. 1), clearly affected cell motility (Table 1) and colony size on LB medium (data not shown), suggesting that YcdT is produced in an active form in strains carrying the pYcdT plasmid. 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 (Fig. 2, last row).
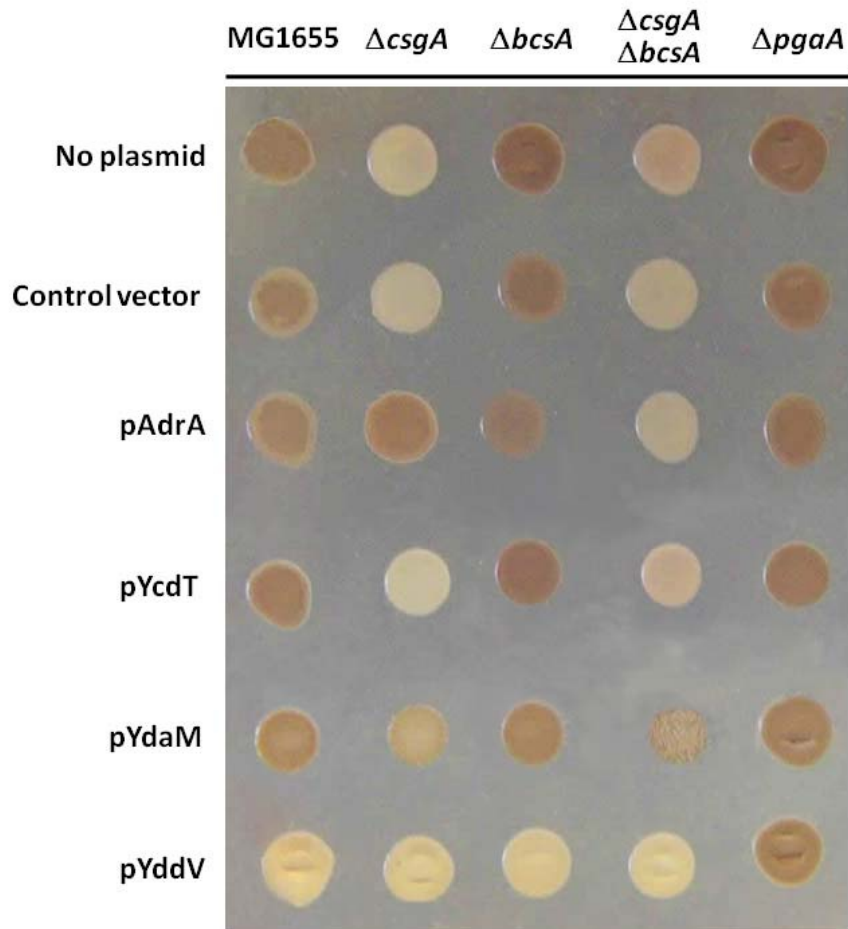
Figure 2. Congo red binding assay. The MG1655 strain and isogenic mutants deficient in production of cell surface-associated structures were transformed with either the pGEM-T Easy vector or the vector carrying the DGC-encoding genes AdrA, YcdT, YdaM and YddV. Strains tested were: MG1655 (WT); Δ*csgA*: 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).

Although a white CR phenotype could indicate negative regulation of curli production by YddV, the observation that YddV-dependent white colony phenotype on CR medium requires a functional *pgaA* gene suggests that YddV overexpression might trigger PNAG overproduction. Indeed, in curli-producing 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 fibers (Gualdi *et al.*, 2008; Ma & 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 absence and in the presence of the pYddV plasmid by plating on agar medium supplemented with Calcofluor, a fluorescent dye able to bind EPS. Presence of pYddV promotes Calcofluor

binding, which is however abolished in the *pgaA* mutant strain AM56, indicating that YddV overexpression increases EPS production in a manner dependent on the presence of a functional *pgaA* gene (Fig. 3, left panel).
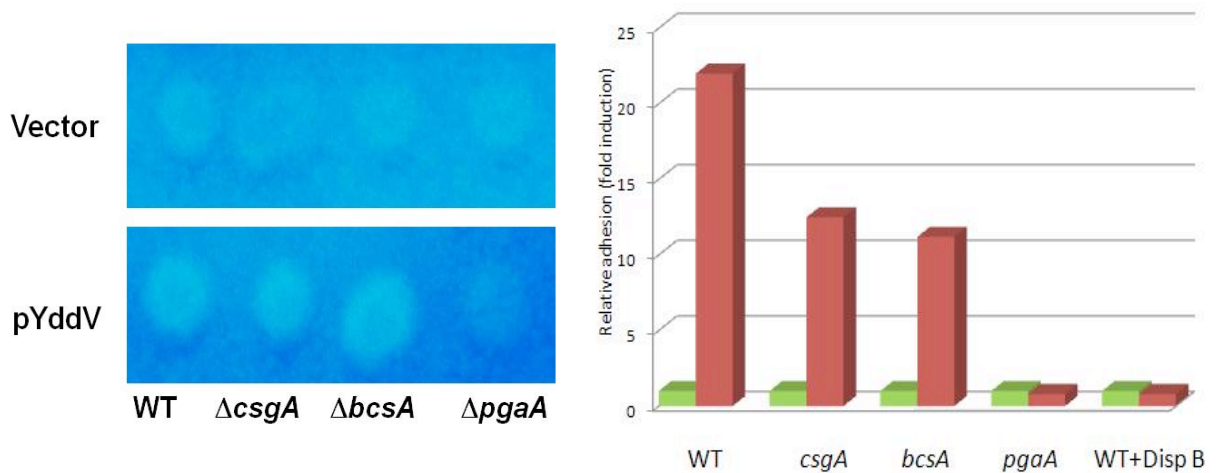

Figure 3. Left panel. Effects of YddV overexpression on EPS production determined by Calcofluor binding assay. The following strains: MG1655 (WT); Δ*csgA*: AM70 (curli-deficient mutant); Δ*bcsA*: LG26 (cellulose-deficient mutant); Δ*pgaA*: AM56 (PNAG-deficient mutant) were transformed either with the control vector (panel above) or with pYddV (panel below). **Right panel**. 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; AM73 (*csgA/bcsA*)=1.2; AM56 (*pgaA*)=3.8, WT+Dispersin B=4.4. Experiments were repeated three times with very similar results.

We determined YddV stimulation of surface adhesion in MG1655 and in its mutant derivatives deficient in production of specific cell surface-associated factors. As shown in Fig. 3 (right panel), 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 (Fig. 3, right panel).

In contrast to YddV, overexpression of either AdrA or YcdT resulted in little or no increase in surface adhesion (Fig. 4). Finally, YdaM overexpression stimulated PNAG production: indeed, YdaM-dependent biofilm formation was affected (ca. 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 (Fig. 4).
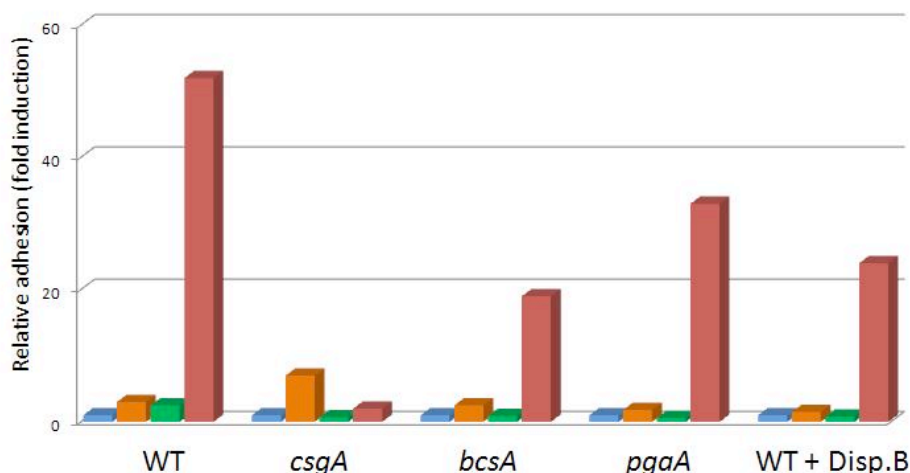

Figure 4. Surface adhesion on polystyrene microtiter plates by strains carrying the pGEM-T Easy control vector (blue bars), pAdrA (orange), pYcdT (green), and pYdaM (red). Surface adhesion values are set to 1 for strains transformed with the control vector. Actual values were: MG1655 (WT)=5.6; AM70 (*csgA*)=1.1; 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.

Regulation of *pgaABCD* expression by DGCs. Regulation of EPS production by DGCs can take place at different levels: cellulose production is stimulated by AdrA through allosteric activation of the cellulose synthase protein machinery (Zogaj *et al.*, 2001; Simm *et al.*, 2004); the YdeH protein affects PNAG production through stabilization of the PgaD protein (Boehm *et al.*, 2009); finally, the YdaM protein activates curli and cellulose production via up-regulation of *csgDEFG* transcription (Weber *et al.*, 2006). We tested the possibility that the YddV protein might regulate PNAG production by affecting transcription of the *pgaABCD* operon, encoding the proteins involved in PNAG biosynthesis. To this aim, we performed quantitative Real Time PCR experiments in MG1655 transformed with pYddV and determined transcript levels of the *pgaA* gene.

As shown in Fig. 5, *pgaA* transcript levels were increased by roughly 10-fold by YddV overexpression. In contrast, overexpression of AdrA and YcdT did not lead to any significant increase in *pgaA* transcript levels. Interestingly, YdaM overexpression also resulted in an increase in *pgaA* transcript levels,

albeit lower than what observed for YddV, consistent with YdaM-dependent stimulation of PNAG production (Fig. 5).
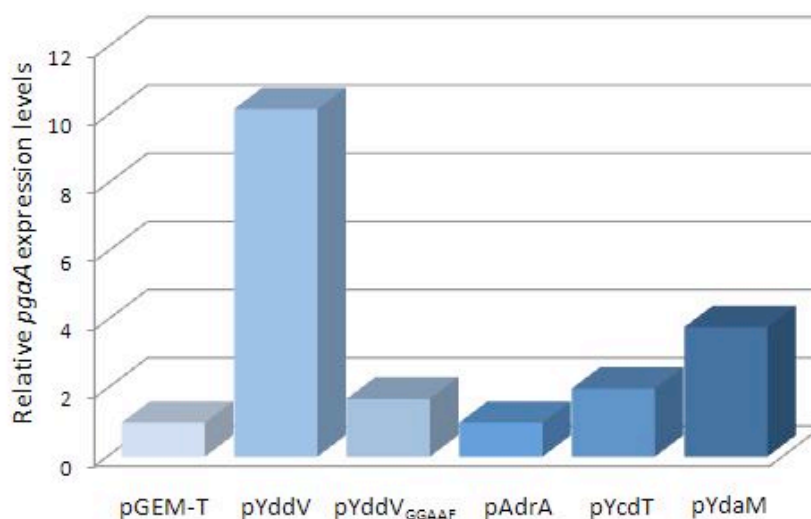

Figure 5. Effects of DGC overexpression on *pgaA* transcript levels. The MG1655 strain was transformed either with the pGEM-T Easy vector or with the following plasmids: pYddV, pYddV$_{GGAAF}$, pAdrA, pYcdT, and pYdaM. The pYddV plasmid carries a copy of the wild type *yddV* allele, while pYddV$_{GGAAF}$ 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 M9Glu/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 5%.

To test if YddV- dependent activation of *pgaABCD* transcription is mediated by its DGC activity, we constructed a plasmid carrying a mutant *yddV* allele encoding a protein in which the amino acids in the GGDEF catalytic site are changed to GGAAF (YddV$_{GGAAF}$); this mutation results in loss of DGC activity (De *et al.*, 2008; Antoniani *et al.*, 2010; data not shown). Overexpression of the YddV$_{GGAAF}$ protein did not affect *pgaA* transcript levels in Real Time PCR experiments (Fig. 5), suggesting that *pgaABCD* regulation by YddV requires its DGC activity.

The *yddV* gene positively controls *pgaABCD* expression and PNAG production. To test if 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). The 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 (Δ*yddV*CTD::*cat*, Table 2). We measured the effects of the Δ*yddV*CTD::*cat* mutation on levels of *pgaA* transcript by Real-Time PCR, which showed that partial deletion of the *yddV* gene resulted in a ca. 3.5-fold reduction in *pgaA* transcript levels in comparison to MG1655 (Fig. 6). In contrast, no detectable reduction was observed in the MG1655*ydaM* mutant AM89, suggesting that the *ydaM* gene is not crucial for *pgaABCD* expression (Fig. 6).
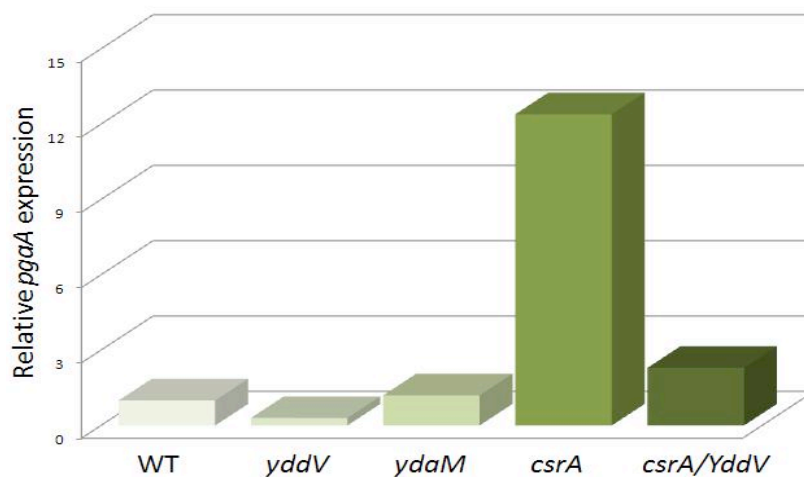
Figure 6. Relative expression levels of the *pgaA* gene in strains MG1655 (WT), AM95 (*yddV*), AM89 (*ydaM*), LT24 (*csrA*) and AM98 (*csrA/yddV*), as measured by Real-Time PCR experiments. *pgaA* expression values in MG1655 (corresponding to a ΔC_t relative to 16S rRNA=15.7) was set to 1. Data are the 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%.

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 (Fig. 6); disruption of the *pgaA* gene, involved in PNAG biosynthesis, counteracts the effects of the *csrA* mutation (Fig. 6), indicating that increased biofilm formation in the *csrA*

derivative of MG1655 depends solely on PNAG production. Partial deletion of the *yddV* gene abolished surface adhesion in MG1655*csrA* (Fig. 7), consistent with reduced *pgaABCD* expression in the MG1655*csrA/yddV* mutant (Fig. 6). Mutations either in the *pgaA* or the *yddV* genes resulted in a 2.5-fold reduction in surface adhesion in the MG1655 background, in agreement with previous observations (Wang *et al.*, 2004; Tagliabue *et al.*, 2010).
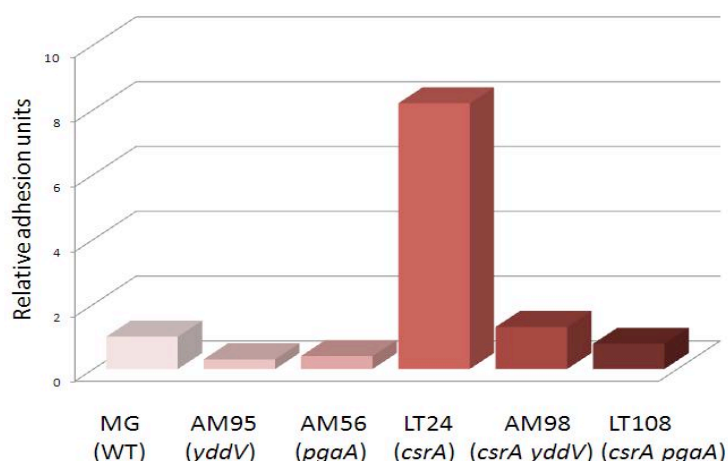
Figure 7. Surface adhesion on polystyrene microtiter plates of strains MG1655 (WT), AM95 (*yddV*), AM56 (*pgaA*), LT24 (*csrA*), AM98 (*csrA/yddV*) and LT108 (*csrA/pgaA*). Surface adhesion value for MG1655 (4.9 in this set of experiments) was set to 1. Results are the average of three independent experiments and standard deviations were always lower than 10%.

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 the wild type copy of the *yddV* gene, or pYddV$_{GGAAF}$, expressing the 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 ability to form biofilm to AM98 (Fig. 8). 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 (Fig. 8), strongly suggesting that YddV-dependent increase in biofilm formation depends on PNAG production.
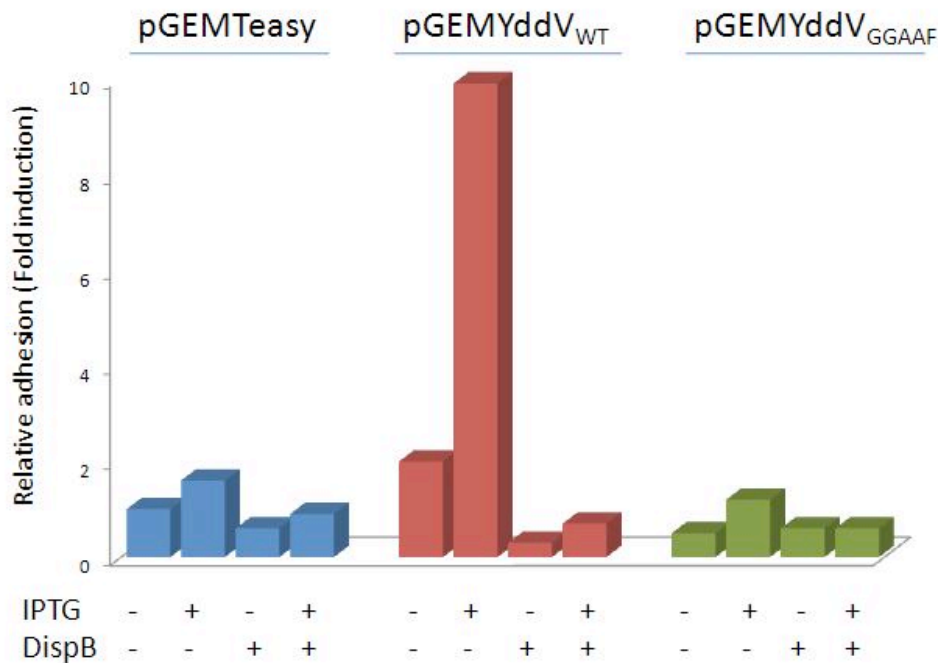
Figure 8. Surface adhesion on polystyrene microtiter plates of strain AM98 (*csrA/yddV*) transformed either with pGEM-T Easy (control vector) or with plasmids carrying *yddV* alleles. The pYddV plasmid carries a copy of the wild type *yddV* allele, while pYddV$_{GGAAF}$ carries a mutant *yddV* allele 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.

Regulation of PNAG-biosynthetic genes by *yddV*. 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 post-transcriptional level, via negative control by the RNA-binding CsrA protein (Wang *et al.,* 2004; Wang *et al.*, 2005; Cerca and Jefferson, 2008); CsrA negatively controls *pgaABCD* expression through binding to a 234-nucleotide untranslated region (UTR) in its mRNA, thus blocking its translation and stimulating its degradation (Wang *et al.*, 2005). We investigated whether the *yddV* gene can positively affect *pgaABCD* expression by increasing transcription initiation from its promoter or at post-transcriptional level via its UTR. To this aim, we constructed two reporter plasmids in which we cloned either the *pgaABCD* promoter region including the 233-nt UTR (-116 to +233 nt relative to the *pgaABCD* transcription start site, defined as "*pgaA*$_{WT}$" regulatory region) or the *pgaABCD* promoter region alone (-116 to +23 nt relative to the *pgaABCD* transcription start site, defined as

"*pgaA*$_{\Delta UTR}$" regulatory region). The two regulatory regions were placed upstream of *Vibrio harveyi* luciferase-encoding *luxAB* genes, to produce respectively the pPgaA$_{WT}$ and pPgaA$_{\Delta UTR}$ plasmids, which were used to transform MG1655 and its *csrA*, *yddV*, and *csrA/yddV* mutant derivatives. Luciferase assays using the pPgaA$_{WT}$ plasmid mirrored the results of Real Time PCR experiments, further confirming that *pgaABCD* expression is dependent on *csrA* inactivation and that it is positively regulated by *yddV* (Fig. 9). In contrast, high levels of luciferase activity from the pPgaA$_{\Delta UTR}$ plasmid were detected in all four strains tested, indicating that deletion of *pgaABCD* UTR by-passes both *csrA*- and *yddV*-dependent regulation (Fig. 9), and strongly suggesting that positive regulation by the YddV protein might take place at the post-transcriptional level.
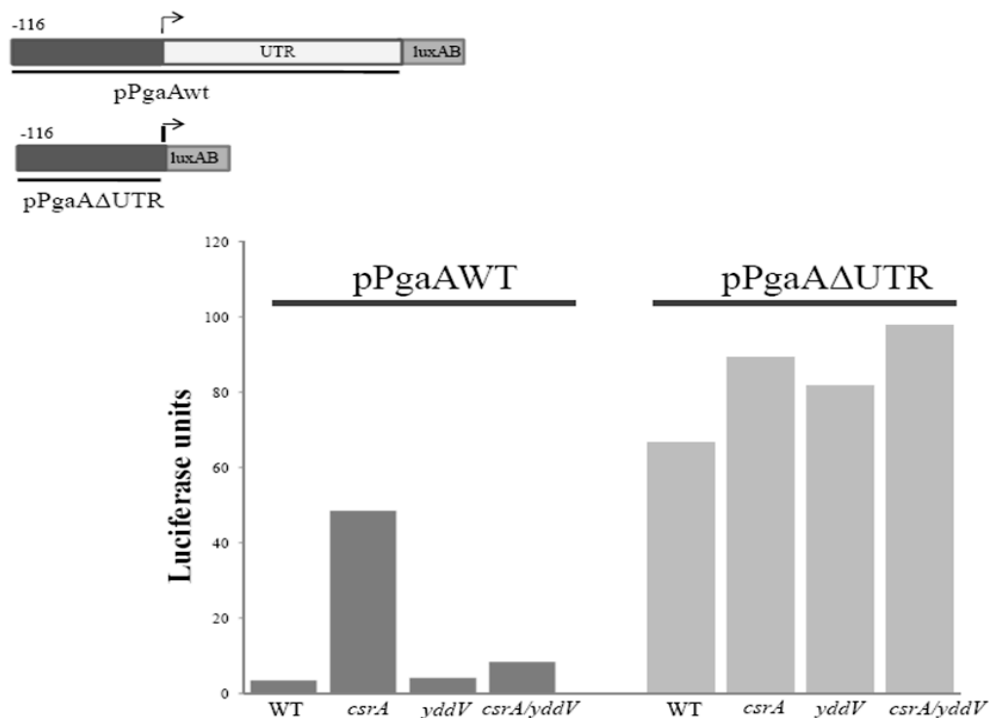
Figure 9. Luciferase assays using pPgaA$_{WT}$ and pPgaA$_{\Delta UTR}$ plasmids (constructs shown in the inset, top left) in strains MG1655 (WT), LT24 (*csrA*), AM95 (*yddV*) and AM98 (*csrA/yddV*). Samples were taken from cultures in stationary phase (OD$_{600nm}$~1.6). Data are the average of two independent experiments with very similar results.

To test whether the ΔGGDEF*yddV* mutation can negatively affect *pgaABCD* mRNA stability, we performed mRNA decay kinetics experiments on the *pgaA* transcript. In both the MG1655 and MG1655*yddV* strains the *pgaA* transcript has a half-life of 1.5 minutes, indicating that *yddV* does not affect

pgaABCD mRNA stability in the MG1655 background (Fig. 10), consistent with lack of any significant difference in *pgaA* transcription in the two strains (Fig. 6). In contrast, inactivation of the *csrA* gene leads to an increase of *pgaA* transcript half-life to 4.2 minutes; the 2.8-fold increase in *pgaABCD* mRNA stability measured in the MG1655*csrA* strain is in very good agreement with previous observations (Wang *et al.*, 2005). In the MG1655*csrA/yddV* double mutant, however, *pgaA* transcript half-life is reduced to 2 minutes (Fig. 10), thus suggesting that the YddV protein positively affects *pgaABCD* mRNA stability.
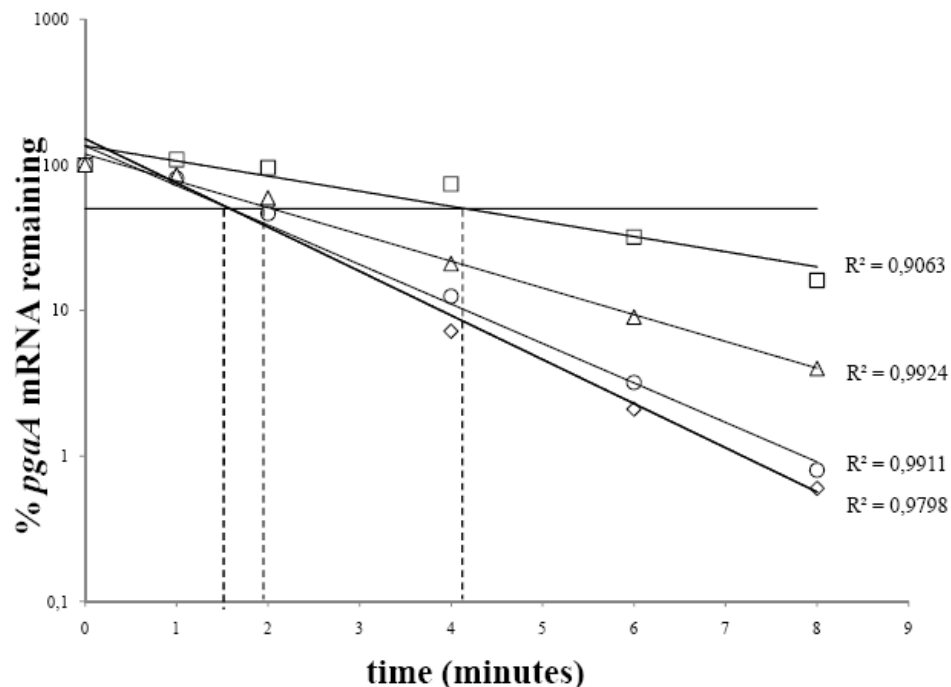
Figure 10. Decay of *pgaA* mRNA as determined by Real Time PCR. Diamonds, MG1655 (WT); circles, AM95 (*yddV*); squares, LT24 (*csrA*); triangles, AM98 (*csrA/yddV*). The solid horizontal line represents the 50% value; the dotted lines show the times corresponding to the mRNA half-lives in the different strains. Correlation coefficients (R^2) for linear interpolation are shown for each set of data and were always >0.9.

Effects of the c-di-GMP phosphodiesterase Dos on *pgaABCD* expression. The *yddV* gene is transcribed in an operon with the *dos* (*yddU*) gene (Mendez-Ortiz *et al.*, 2006); the product of the *dos* gene is a heme-binding oxygen sensor (Delgado-Nixon *et al.*, 2000), which possesses putative domains for both DGC and c-di-GMP phosphodiesterase (PDE) activity (Schmidt *et al.*, 2005). However, due to degeneration of the GGDEF motif responsible for

DGC catalytic activity, Dos can only function as a PDE (Schmidt *et al.*, 2005; Tuckerman *et al.*, 2009). The presence in the same transcriptional unit of genes coding for a DGC and a PDE suggests that Dos might modulate YddV DGC activity. Indeed, a recent report shows that the two proteins co-purify and form a complex in solution (Tuckerman *et al.*, 2009), suggesting that the YddV-Dos protein complex might exist in a stable form in the bacterial cell. Environmental signals might modulate either the DGC activity of YddV or the PDE activity of Dos. Since the insertion of the cloramphenicol resistance cassette into the *yddV* gene could result in polar effects on *dos* expression, we compared *dos* transcript levels in the MG1655Δ*yddV*CTD*::cat* strain to MG1655 by Real Time-PCR. Transcription of the *dos* gene was only reduced by ca. 2.5-fold in the MG1655*yddV* strain (data not shown), suggesting that in this strain the *dos* gene is still expressed at significant levels, probably due to transcription readthrough from the promoter of the cloramphenicol resistance cassette upstream of the *dos* gene. To investigate the possible role of *dos* in *pgaABCD* regulation, we inactivated the *dos* gene both in the MG1655 strain and in its *csrA* mutant derivative. Real-Time PCR experiments confirmed that *dos* inactivation increased *pgaA* transcript levels both in the MG1655 (ca. 4-fold) and in the MG1655*csrA* strains (ca. 2-fold; Fig. 11), consistent with the hypothesis that Dos modulates DGC activity by the YddV protein.
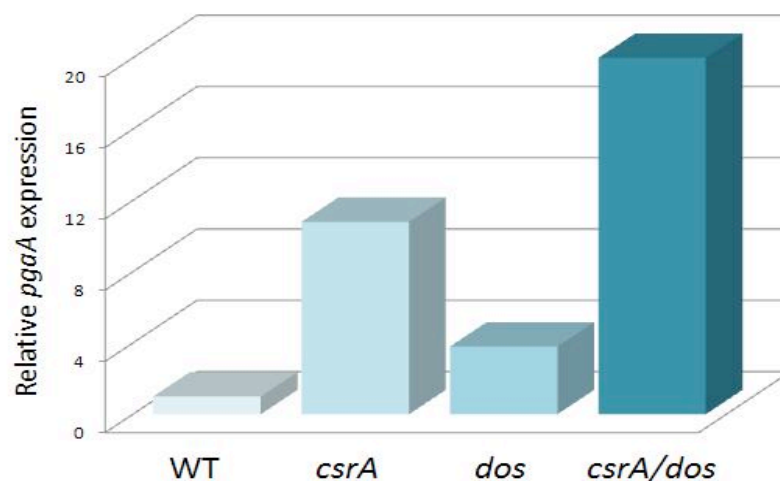
Figure 11. Relative expression levels of the *pgaA* gene in strains MG1655 (WT), LT24 (*csrA*), AM109 (*dos*), and LT110 (*csrA/dos*), as measured by Real-Time PCR experiments. *pgaA* expression values in MG1655 (corresponding to a Δ*Ct* relative to 16S rRNA=15.5) was set to 1. Results are the average of three independent experiments, each performed in triplicate, with very similar results.

DISCUSSION

In Enterobacteria, biosynthesis of the c-di-GMP signal molecule by diguanylate cyclases (DGCs) stimulates the transition from planktonic to biofilm 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 overexpression of YddV, a DGC protein, promotes production of the EPS poly-*N*-acetyl (ß-1,6) glucosamine (PNAG; Fig. 2, 3) by activating expression of *pgaABCD*, the PNAG biosynthetic operon (Fig. 3). *pgaABCD* activation and consequent stimulation of PNAG biosynthesis requires DGC activity by the YddV protein (Fig. 5, 8); however, increase of intracellular c-di-GMP due to overexpression of other DGCs, such as AdrA and YcdT, is not sufficient to activate PNAG production (Fig. 1, 2, 4). In contrast, overexpression of YdaM, a cytoplasmic DGC, resulted in increased PNAG production (Fig. 4) and *pgaABCD* expression (Fig. 4, 5), although to a lesser degree than YddV. However, unlike *yddV*, *ydaM* inactivation did not affect *pgaABCD* expression (Fig. 5), suggesting specific dependence of this process on the YddV protein. Specificity of DGCs-mediated regulation might indicate that c-di-GMP biosynthesis is needed to trigger specific protein-protein (or protein-DNA, or protein-RNA) interactions between DGCs and their targets (Hengge, 2009). Thus, it can be speculated that c-di-GMP biosynthesis could act as an activating step in signal transduction pathways leading to regulation of gene expression and of protein activity.

Dependence of PNAG production on c-di-GMP biosynthesis has already been described both in *Yersinia pestis*, where the HmsT protein activates PNAG production by allosteric activation of its biosynthetic machinery (Kirillina *et al.*, 2004). In contrast, our results suggest that the YddV protein promotes PNAG production by activating the expression of the PNAG biosynthetic operon *pgaABCD* (Fig. 5, 6), possibly via interaction with a c-di-GMP-responsive regulatory protein. In addition to YddV, PNAG production is controlled by another DGC, YdeH, which positively affects PgaD protein stability via a yet unknown mechanism (Boehm *et al.*, 2009). Similarly, cellulose biosynthesis is regulated by DGC proteins at both gene expression and protein activity levels: the YdaM protein positively regulates *csgDEFG* transcription (Weber *et al.*, 2006); the CsgD protein, in turn, activates *adrA* transcription. The *adrA* gene

encodes another DGC that stimulates cellulose production through allosteric activation of the cellulose synthase machinery (Romling *et al.*, 2000; Zogaj *et al.*, 2001). Thus, it appears that DGC-dependent control at multiple levels is a common mechanism for EPS biosynthesis regulation in *E. coli*. A model summarizing multiple level EPS regulation by DGCs is summarized in Fig. 12. Recent observations indicate that c-di-GMP can act as a riboswitch, binding specific elements (aptamers) in the untranslated regions (UTR) in some mRNAs and affecting their stability (Sudarsan *et al.*, 2008). The *pgaABCD* transcript is characterized by a rather long UTR (234nt, Wang *et al.*, 2005) and is regulated at the level of mRNA stability by the CsrA protein; our results show that a functional *yddV* gene is able to prevent degradation of *pgaABCD* transcript (Fig. 10). This effect cannot be detected in the wt strain, where the presence of the CsrA protein might override mRNA stabilization by YddV, but it can only be detected in a MG1655*csrA* background. Thus our findings suggest that the YddV protein might regulate gene expression by affecting mRNA stability in *E. coli*, in line with recent observations that c-di-GMP can regulate gene expression through direct binding to riboswitch elements in mRNAs (Sudarsan *et al.*, 2008): it is likely that, in the bacterial cell, mRNA/c-di-GMP interaction might be mediated by protein factors, possibly DGCs themselves, in order to ensure specific gene expression regulation. Indeed, effects on RNA stability have already been shown for another protein carrying a GGDEF domain, the CsrD protein (Suzuki *et al.*, 2006). However, CsrD is unable to synthesize c-di-GMP due to lack of conservation of the catalytic site (Suzuki *et al.*, 2006); thus, the YddV protein might be the first example of a DGC being able to regulate gene expression by affecting mRNAs stability, either directly or via a YddV-dependent factor. We speculate that YddV-mediated mRNA stabilization takes place through interaction with the 233-nt UTR of the *pgaABCD* transcript (Fig. 9), possibly promoting the formation of a secondary structure resulting in mRNA stabilization. Future investigations will allow the identification of the specific nucleotide sequences involved in YddV-dependent mRNA stabilization and elucidation of its molecular mechanism.
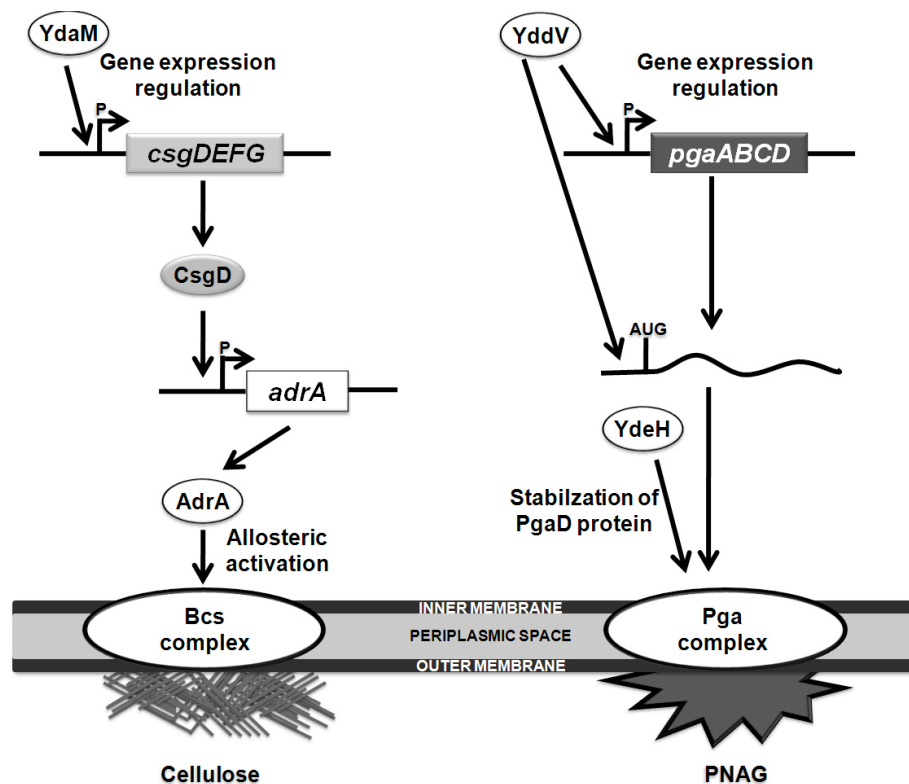
Figure 12. Model summarizing transcriptional and post-transcriptional regulation of EPS biosynthesis by DGC proteins. Proteins with DGC activity are indicated in shaded ellipses. Cellulose biosynthesis, represented on the left hand side of the figure, is regulated by YdaM, promoting transcription of the *csgD* gene (Weber *et al*., 2006), and AdrA, which activates cellulose synthase activity by the cellulose synthase (Bcs) complex through its DGC activity (Romling *et al.*, 2000; Zogaj *et al.*, 2001). PNAG production is positively affected by YddV through activation of *pgaABCD* transcription or post-transcriptionally via its UTR (see Fig. 5, 6, 10) and by YdeH-dependent stabilization of the PgaD protein (Boehm *et al.*, 2009) at post-transcriptional level.

METHODS

Bacterial strains and growth conditions. Bacterial strains used in this work are listed in Table 2. When not otherwise stated, bacteria were grown in M9Glu/sup (M9 inorganic salts (Smith and Levine, 1964), 5 g/L glucose, 0.25 g/L Peptone, 0.125 g/L Yeast Extract). When needed, antibiotics were used at the following concentrations: ampicillin, 100 µg/ml; chloramphenicol, 50 µg/ml; kanamycin, 50 µg/ml; tetracycline, 25 µg/ml; rifampicin, 100 µg/ml. For Congo red (CR) or Calcofluor (CF) assays, overnight cultures were spotted, using a replicator, on agar media supplemented with 0.5% Casamino acids, 0.15% yeast extract, 0.005% MgSO$_4$, 0.0005% MnCl$_2$; either 0.004% Congo red

and 0.002% Coomassie blue (for CR medium) or 0.005% Calcofluor (for CF medium) were added after autoclaving. Bacteria were grown for 20h at 30° C; phenotypes were better detectable after 24-48h incubation at 4° C.

Biofilm formation assays. Biofilm formation in microtiter plates was determined essentially as described (Dorel *et al.*, 1999). Bacterial cultures were grown overnight in M9Glu/sup at 30°C in polystyrene microtiter plates (0.2 ml); cell density of the culture was determined spectrophotometrically at 600nm (OD_{600nm}). Cells attached to the microtiter plates were washed gently with water and stained for 20 min with 1% crystal violet in water (CV), thoroughly washed with water and dried. For semi-quantitative determination of biofilms, CV-stained cells were resuspended in 0.2 ml of 95% ethanol by vigorous pipetting. The OD_{600nm} of each sample was determined and normalized to the OD_{600nm} of the corresponding liquid cultures (Adhesion units). Sensitivity of biofilms to treatment with the PNAG degrading enzyme Dispersin B (Kaplan *et al.*, 2004; purchased from Kane Biotech Inc., Winnipeg, Canada) was performed by adding 20 µg/ml of the enzyme to the growth medium.

Plasmid construction. Plasmids used in this work are listed in Table 2. For overexpression of genes encoding DGCs, genes of interest were amplified by PCR and the corresponding products cloned into the pGEM-T Easy vector. Correct orientation of the inserts (*i.e.*, under the control of the P*lac* promoter) was verified by PCR using primers listed in Table 3. For DGC-overproduction studies, strains carrying pGEM-T Easy derivatives were grown at 30°C in M9Glu/sup medium in the absence of IPTG induction of the P*lac* promoter. The pYddV$_{GGAAF}$ plasmid, carrying the *yddV* gene mutated in the DGC catalytic site, was obtained by 3-step PCR mutagenesis (Li & Shapiro, 1993) using the primers listed in Table 3. All constructs were verified by sequencing.

Luciferase assay. Luciferase assays were performed as described below, using the vector pJAMA8 (Jaspers *et al.*, 2000), which carries promoterless *luxAB* genes from *Vibrio harveyi*. 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 deleted (ΔUTR, ranging from -116 to +23 nucleotides relative to the *pgaABCD* mRNA start site) were amplified from the chromosomal DNA using primers including *SphI* and the *XbaI* restriction sites and cloned into the multiple cloning site of pJAMA8 to obtain pPgaA$_{WT}$ and pPgaA$_{ΔUTR}$, respectively. Bacterial strains containing the different reporter plasmids were grown overnight. The samples were adjusted to an OD_{600} of 0.05–0.1 in PBS buffer. 20 µl of this solution was tested for luciferase activity by adding 200 ml PBS containing n-decanal to a final concentration of 2 nM. Measurement of relative light units (RLU) was

conducted by a 2 s pre-measurement delay followed by a 3 s measurement after addition of the substrate in a MicroLumat LB 96 P luminometer (Berthold Technologies). Results are expressed as RLU per OD_{600} of the tested bacterial samples.

Gene expression studies. Real-Time PCR for determination of relative expression levels was performed on overnight cultures grown in M9Glu/sup medium at 30° C. Primers for Real- Time PCR are listed in Table 3. RNA extraction and further Reverse Transcription and cDNA amplification steps were performed as described (Gualdi *et al.*, 2007), using 16S RNA as reference gene. mRNA stability was measured by Real-Time PCR experiments in the presence of rifampicin as described (Wang *et al.*, 2005).

Other methods. *E. coli* MG1655 mutant derivatives were constructed either using the λ Red technique (Datsenko & Wanner 2000) or by bacteriophage P1 transduction (Miller, 1972), except the AM89 strain (MG1655 *ydaM*::*Tn5-kan*) 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. Bacterial cell motility was evaluated as described (Pesavento *et al.*, 2008). Determination of intracellular c-di-GMP concentration was performed as previously described (Antoniani *et al.*, 2010).

Table 2. *E. coli* strains and plasmids used in this work.

E. coli strains	Relevant genotype or characteristics	Reference
MG1655 K-12,	K-12, F⁻ λ⁻ rph-1	Blatter *et al.*, 1997
AM56	MG1655Δ*pgaA::cat*	This work
AM70	MG1655Δ*csgA::cat*	This work
LG26	MG1655Δ*bcsA::kan*	Gualdi *et al.*, 2008
AM73	MG1655Δ*csgA::cat,* Δ*bcsA::kan*	This work
AM89	MG1655*ydaM::Tn5-kat*	This work
AM95	MG1665*yddVCTD::cat* (*yddVΔ$_{931-1383}$::cat*)	Tagliabue *et al.*, 2010
AM98	MG1655*csrA*::Kan,*yddVΔ$_{931-1383}$::cat*	This work
AM109	MG1655*dos::tetΔ$_{1200-2004}$*	This work
LT24	MG1655*csrA::kan*	This work
LT108	MG1655*csrA::kan* Δ*pgaA::cat*	This work
LT110	MG1655*csrA::kan, dos::tetΔ$_{1200-2004}$*	This work

Plasmids

pGEM-T Easy	Control vector allowing direct cloning of PCR products, ampicillin resistance	Promega
pAdrA	*adrA* gene cloned as PCR product into pGEM-T vector	This work
pYcdT	*ycdT* gene cloned as PCR product into pGEM-T vector	This work
pYdaM	*ydaM* gene cloned as PCR product into pGEM-T vector	This work
pYddV	*yddV* gene cloned as PCR product into pGEM-T vector	Tagliabue *et al.*, 2010
pYddV$_{GGAAF}$	*yddV* allele carrying mutation resulting in GGDEF→GGAAF change in the DGC catalytic site of the YddV protein	Tagliabue *et al.,* 2010
pJAMA8	Control vector fo luciferase assays, ampicillin resistance	Jaspers *et al.*, 2000
pPgaA$_{WT}$	*pgaA* promoter and regulatory region (-116 to +233 relative to transcription start site) cloned into the *SphI/XbaI* sites of pJAMA8	This work
pPgaA$_{ΔUTR}$	*pgaA$_{ΔUTR}$* (-116 to + 23 relative to transcription start site) cloned into the *SphI/XbaI* sites of pJAMA8	This work

Table 3. Primers used in this work.

Primers	Sequence	Utilization
adrA_for	5'-GCTCCGTCTCTATAATTTGGG-3'	*adrA* cloning
adrA_rev	5'-ATCCTGATGACTTTCGCCGG-3'	*adrA* cloning
ydaM_for	5'-GCGATCGGATAGCAACAA-3'	*ydaM* cloning
ydaM_rev	5'-GAAGTCGTTGATCTCGAC-3'	*ydaM* cloning
ycdT_for	5'-GGGATCTACAACCTACAG-3'	*ycdT* cloning
ycdT_rev	5'-CATATTACGTGGGTAGGATC-3'	*ycdT* cloning
yddV_for	5'-CCAGCCTTATAAGGGTGTG-3'	*yddV* cloning and mutant screening
yddV_rev	5'-TTACCTCTGCATCCTGGC-3'	*yddV* cloning
ydaM_for	5'-GCGATCGGATAGCAACAA-3'	*ydaM* mutant screening
ydaM_rev	5'-GAAGTCGTTGATCTCGAC-3'	*ydaM* mutant screening
EZ-Tn5_for	5'-CCTCTTTCTCCGCACCCGAC-3'	*ydaM* mutant screening
yddV$_{GGAAF}$_for	5'-TACGGGGGCG**C**TG**C**ATTTATCATT-3'	Construction of pYddV$_{GGAAF}$
yddV$_{GGAAF}$_rev	5'-AATGATAAAT**G**CA**G**CGCCCCCGTA-3'	Construction of pYddV$_{GGAAF}$
csgA_cat_for	5'-TTTCCATTCGACTTTTAAATCAATCCGATGGGGGTTTTACTACCTGTGACGGAAGATCAC-3'	*csgA* inactivation
csgA_cat_rev	5'-AACAGGGCTTGCGCCCTGTTTCTGTAATACAAATGATGTAGGGCACCAATAACTGCCTTA-3'	*csgA* inactivation
dos_tet_for	5'CCTGCACAATTACCTCGATGACCTGGTCGACAAAGCCGTCCTAGACATCATTAATTCCTA-3'	*dos* inactivation
dos_tet_rev	5'GTTAAATGAAAACCCGCGAGTGCGGGCGAGAGGAATTTGGAAGCTAAATCTTCTTTATCG-3'	*dos* inactivation
yddV_cat_for	5'-GGATGTACTGACGAAATTACTTAACCGCCGTTTCCTACCGTACCTGTGACGGAAGATCAC-3'	*yddV* inactivation
yddV_cat_rev	5'-CATCGGTTAGCTTCATGATTACCTCTGCATCCTGGGGAGTAATACAGGTACCTGTGACGGAAGATCAC-3'	*yddV* inactivation
pgaA_cat_for	5'-ATACAGAGAGAGATTTTGGCAATACATGGAGTAATACAGGTACCTGTGACGGAAGATCAC-3'	*pgaA* inactivation
pgaA_cat_rev	5'-ATCAGGAGATATTTATTTCCATTACGTAACATATTTATCCGGGCACCAATAACTGCCTTA-3'	*pgaA* inactivation
cat_rev	5'-GGGCACCAATAACTGCCTTA-3'	Mutant verification
csgA_for	5'-ACAGTCGCAAATGGCTATTC-3'	Mutant verification
pgaA_for	5'-TGGACACTCTGCTCATCATTT-3'	Mutant verification
pPgaA-delUTR _for	5'- GCATGCAACAATTAAATCCGTGAGTGCCG-3'	*pgaA* promoter cloning
pPgaA-delUTR_rev	5'- TCTAGAATCTTCAGGAATACGGCATAAAT-3'	*pgaA* promoter cloning
pPgaA_wt_for	5'- AGCATGCCTCAAATAGTCTTTTTCCAT-3'	*pgaA* promoter cloning

Primers	Sequence	Utilization
pPgaA_wt_rev	5'- ATCTAGATACATCCTGTATTACTCCATG-3'	*pgaA* promoter cloning
16s_for	5'-TGTCGTCAGCTCGTGTCGTGA-3'	qRT-PCR
16s_rev	5'-ATCCCCACCTTCCTCCGGT-3'	qRT-PCR
dos_RT_for	5'-CAGAGAAGCTCTGGGGATACA-3'	qRT-PCR
dos_RT_rev	5'-TTTTTCTCCAGCTGCAGCTCC-3'	qRT-PCR
pgaA_RT_for	5'-CCGCTACCGTCATCAGCAATT-3'	qRT-PCR
pgaA_RT_rev	5'-AGCGCCTTTTGCCACAGTGT-3'	qRT-PCR

STUDY OF PHL1228, A BIOFILM FORMING MUTANT

INTRODUCTION

In *E. coli*, several genes coding for extracellular features are involved in bacterial adhesion to solid surfaces and/or biofilm formation. As I showed in previous chapters, YddV regulates the expression of different adhesion factors; in particular, YddV overexpression leads to production of poly-*N*-acetylglucosamine (PNAG), an EPS able to promote biofilm formation, by triggering expression of *pgaABCD*, the PNAG biosynthetic operon. *pgaABCD* expression is very low in MG1655 laboratory strain, in any tested growth condition. Consistent with this observation, mutations in regulatory genes or in promoters of biofilm-related genes can lead to reversion to a biofilm-forming phenotype. In this work, I investigated the PHL1228 strain (coming from a collaboration with the group of Corinne Dorel), a spontaneous mutant of *E. coli* MG1655 showing a strong biofilm-forming phenotype. PHL1228 was obtained by EB12 strain (a derivative of MG1655 in which the *csgA* gene, encoding the main curli subunit (see Chapter II), has been inactivated by a kanamycin resistance cassette) grown as continuous culture for several hundred generations; EB12 strain, unable to produce curli, was used in order to identify possible determinants for biofilm formation different from curli fibers. Cells appearing to attach to the chemostat glass were isolated and tested for their adhesion properties. Gene array experiments showed that biofilm-formation by PHL1228 is mediated by increased expression of the *pgaABCD* operon; I performed a molecular characterization of the PHL1228 strain, in order to investigate the mutation leading to *pgaABCD* expression.

RESULTS

Global gene expression in PHL1228. PHL1228 strain is a spontaneous mutant of *E. coli* MG1655 showing a strong biofilm-forming phenotype (Fig. 1). Since gene expression regulation in bacteria takes place mostly at the transcriptional level, I expected that biofilm formation by PHL1228 would be due to increased transcription of one or more genes encoding biofilm

determinants. Thus, in order to compare gene expression levels in EB12 and PHL1228, a gene array experiment was carried out on bacteria grown in M9sup medium at 28°C in stationary phase, i.e the optimal conditions for biofilm formation by PHL1228. About 100 genes were found to be up-regulated in PHL1228, while almost 300 genes were down-regulated. Such a high number of differentially regulated genes in PHL1228 would be consistent with a mutation in a global regulatory gene. Among genes differently regulated in PHL1228 (transcriptional regulators, genes involved in general metabolism etc…), I will focus on genes related to outer membrane and cell surface, in order to identify possible adhesion factors. Known outer membrane factors taking part in biofilm formation, such as genes involved in colanic acid synthesis (*wcaCG*), as well as flagellar (*flhEH*, *fliFN*,*flgFH*) and fimbrial (*fimF*) genes, were expressed at lower levels in PHL1228. Results of microarray analysis also pointed to a strong transcription activation of *ompR*-regulated genes (Oshima *et al.*, 2002; Salgado *et al.*, 2004), such as *csgD* (10.7), *ompF* (51), *ompC* (7.7), *nmpC* (9.8), *fepA* (5.3), *osmB* (4.7).
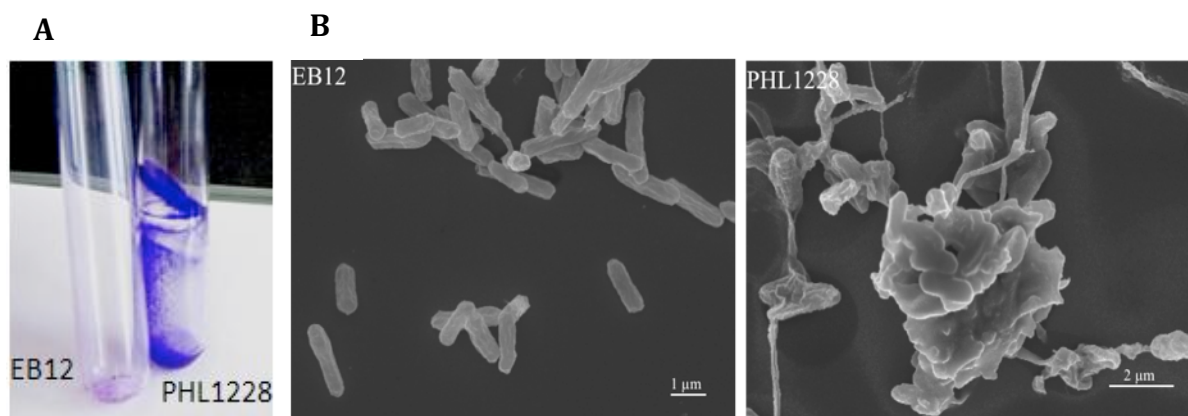
Figure 1. Phenotype of the biofilm forming strain PHL1228. **A)** Cristal violet stained biofilm of PHL1228 compared to its parental strain EB12. **B)** Raster electron micrographs of EB12 and the biofilm-forming PHL1228.

Interestingly, transcription levels of the *pgaABCD* genes were dramatically enhanced in PHL1228 (20- to 200-fold). The *pgaABCD* operon defines a single transcriptional unit on the *E. coli* chromosome; a divergently transcribed gene, *ycdT*, encoding a transmembrane GGDEF protein, was also expressed at high levels (5-fold) in PHL1228. As already mentioned in previous chapters, *pgaABCD* genes show a high degree of similarity to the *hmsHFRS*

operon of *Yersinia pestis*, which also displays an identical gene organization, while the *ycdT* gene is homologous to the regulator protein *hmsT*. The *hms* genes encode a haemin-binding system and is also involved in biofilm formation by *Y. pestis* in the flea midgut (Hinnebusch *et al.*, 1996). The *pgaABCD* operon shows weaker similarity with the *ica* locus, which encodes for polysaccharide intercellular adhesin (PIA), involved in biofilm formation in *Staphylococcus epidermidis*, thus suggesting that it might encode functional biofilm determinants in *E. coli*. Indeed, the *pgaABCD* operon encodes for proteins responsible for the biosynthesis of poly-*N*-acetyl-glucosamine (PNAG; Wang *et al.*, 2004). In order to confirm the results of gene array experiments suggesting strong stimulation of both *pgaABCD* and *ycdT* gene expression, Real-time PCR experiments were performed. Results showed a very good correlation between RT-PCR and gene array experiments (data not shown).

Outer membrane protein analysis. Biofilm determinants are usually exposed on the cell surface, and are often associated to the outer-membrane in Gram negative bacteria. Thus, we performed analysis of the outer membrane proteins (OMPs) fraction of PHL1228 in comparison to EB12 (Fig. 2). 40µg of proteins was loaded onto the gel for either strain.
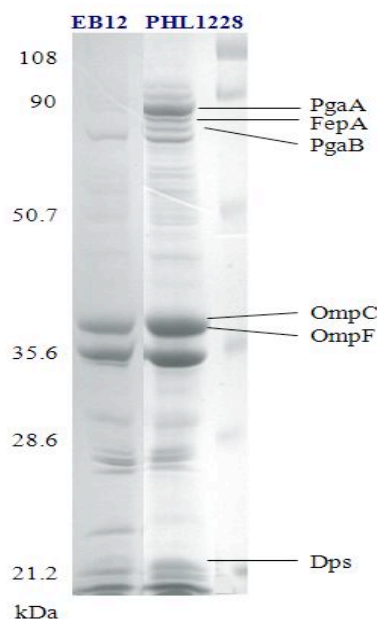
Figure 2. SDS-PAGE analysis of outer membrane protein pattern of EB12 and PHL1228. Proteins identified by mass spectrometry, which differ in expression in PHL1228, are indicated.

Proteins differently expressed in PHL1228 were identified by Mass Spectrometry determination following excision from gel and trypsin digestion. Expression of *ompR*-dependent proteins such as OmpC, OmpF and FepA was much stronger in PHL1228, in agreement with gene array results. Surprisingly, the DNA-binding protein Dps was found in the OMP fraction of PHL1228. It is noteworthy that the Dps protein is also present in significant amounts in the OMP fraction of other biofilm-forming *E. coli* strains, thus suggesting a possible secondary role as an outer membrane component (Landini, 2009; Pham *et al.*, 2010). The PgaA and PgaB proteins were also found in higher amount in the PHL1228 outer membrane fraction; this suggests that PgaA and PgaB are surface-exposed proteins, consisting with their role in biofilm formation.

Expression of the *pgaABCD* operon is responsible for biofilm phenotype. The results of the experiments presented in the previous sections clearly show that the *pgaABCD* operon is strongly expressed in the PHL1228 and the PgaA and PgaB proteins are located in the outer membrane protein fraction (Fig. 2). To establish if *pgaABCD* expression is responsible for the biofilm-forming phenotype of the PHL1228 strain, we targeted the *pgaA* gene for insertion mutagenesis with a cloramphenicol resistance cassette, so obtaining the LT106 strain (PHL1228*pgaA*). Inactivation of *pgaA* results in a sharp decrease in the ability of PHL1228 to form biofilm and to adhere to microtitre plates (Fig. 3), suggesting that PNAG represents the biofilm determinant for PHL1228.
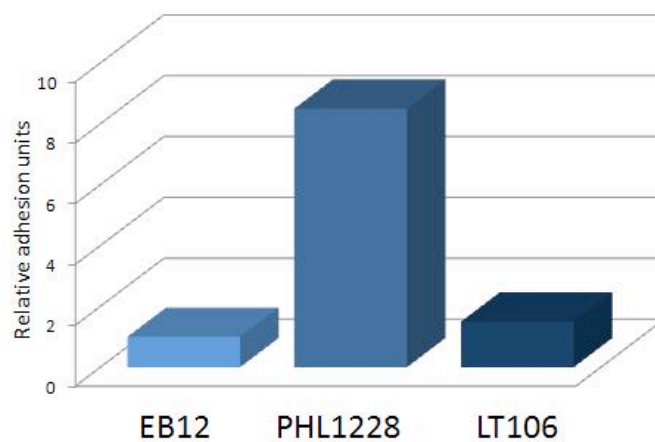
Figure 3. Surface adhesion on polystyrene microtitre plates of EB12 (MG1655*csgA*), PHL1228 and LT106 (PHL1228*pgaA*) strains. Surface adhesion values are set to 1 for EB12. Actual adhesion values were: EB12 (*csgA*): 0.65; PHL1228: 5.5; LT106 (*pgaA*): 0.95.

Study on *pgaABCD* expression. In order to characterize the mutation/s leading to biofilm formation in PHL1228, we decided to focus on *csrA* (Carbon Storage Regulatory A) gene. CsrA is a small RNA-binding protein that recognizes sequences located within the leaders of target mRNAs and alters their translation and stability (Liu *et al.*, 1995; 1997; Wei *et al.*, 2001; Baker *et al.*, 2002; Dubey *et al.*, 2003). Two untranslated RNAs, CsrB and CsrC, antagonize CsrA activity by sequestering this protein (Liu *et al.*, 1997; Weilbacher *et al.*, 2003). CsrA binds to six sites in the *pga* operon leader transcript and one of the CsrA binding sites overlaps the cognate SD sequence. Thus, *csrA* deletion leads to increased *pgaABCD* expression.
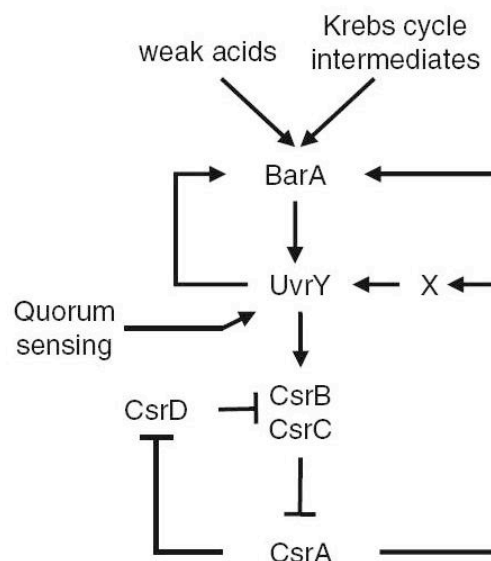
Figure 4. Summary of the regulatory interactions of CsrA/B/C, BarA/UvrY. CsrA activates *csrB*/*csrC* transcription indirectly (Gudapaty *et al.*, 2001). This effect of CsrA requires functional UvrY, which directly activates *csrB* transcription. UvrY also activates the expression of *barA*, in an autoregulatory loop. Finally, CsrB RNA binds to ~18 subunits of CsrA protein and antagonizes its regulatory effects in the cell (Liu *et al.*, 1997; Romeo, 1998).

First of all we verified if, in PHL1228, regulation of *pgaA* transcript occurs at the UTR level performing luciferase assays using two reporter plasmids in which we cloned either the *pgaABCD* promoter region including the 233-nt UTR (-116 to +233 nt relative to the *pgaABCD* transcription start site, defined as "*pgaA*$_{WT}$" regulatory region) or the *pgaABCD* promoter region alone (-116 to +23 nt relative to the *pgaABCD* transcription start site, defined as "*pgaA*$_{\Delta UTR}$" regulatory region). pPgaA$_{WT}$ and pPgaA$_{\Delta UTR}$ plasmids (see Chapter IV) were used

to transform EB12 strain and its spontaneous mutant PHL1228. Luciferase assays with pPgaA$_{WT}$ confirmed that *pgaA* transcript levels are dramatically higher in PHL1228 compared to EB12 strain; in contrast, high levels of luciferase activity from the pPgaA$_{\Delta UTR}$ plasmid were detected both in EB12 and PHL1228, indicating that the deletion of *pgaABCD* UTR it's able to by-pass CsrA regulation and suggesting that the difference between *pgaA* expression in the two strains would be due to a negative regulation at the UTR level (Fig. 5).
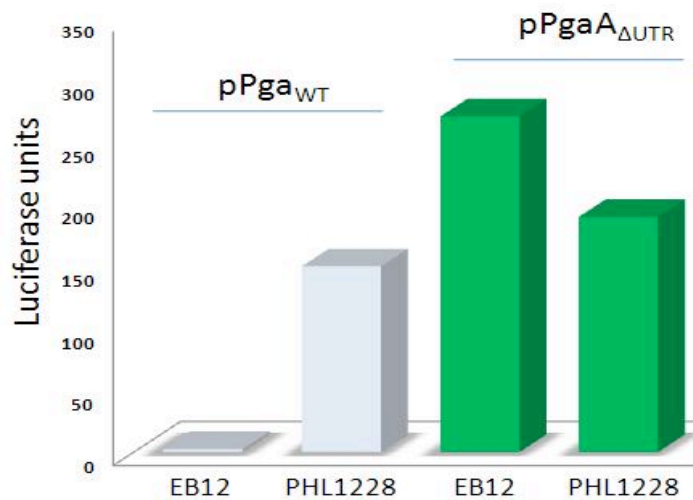
Figure 5. Luciferase assays using pPgaA$_{WT}$ (light blue columns) and pPgaA$_{\Delta UTR}$ (green columns) plasmids in strains EB12 (*csgA*) and PHL1228 (EB12*pgaA*). Samples were taken from cultures in stationary phase (OD$_{600nm}$~1.6). Data are the average of two independent experiments with very similar results.

Since the behavior of PHL1228 strain resembled a *csrA* mutant strain (biofilm formation, high *pgaA* expression, *pgaABCD* transcript regulation at post-transcriptional level), we performed mRNA decay kinetics experiments on the *pgaA* transcript, in order to verify CsrA-dependent mRNA destabilization activity. Cell growth was stopped with rifampicin, samples were taken at different time points after addition of rifampicin and RT-PCR experiments measuring *pgaA* transcript levels were performed. In the EB12 strain the *pgaA* transcript is rapidly degraded (similar to *pgaA* mRNA in the parental strain MG1655; see Chapter IV, Fig. 10; Wang *et al.*, 2005), while it is very stable (half-life longer than 8 minutes) in the PHL1228 strain, similar to previous

results showing *pgaA* mRNA stabilization in a *csrA* mutant strain (Chapter IV, Fig. 10; Wang *et al.,* 2005; Fig. 6).
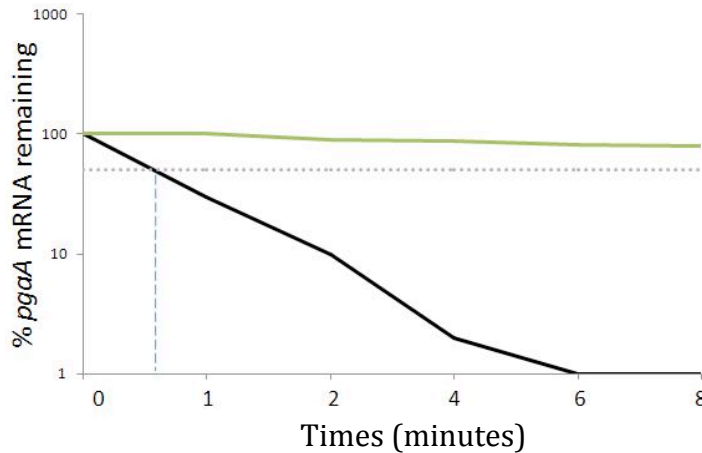
Figure 6. Decay of *pgaA* mRNA as determined by Real Time PCR. Black line, EB12; green line, PHL1228. The dotted horizontal line represents the 50% value; the dotted vertical line shows the time corresponding to the mRNA half-life in EB12. Values are from three different experiments with very similar results.

We decided to investigate if CsrA pathway was somehow deregulated in the biofilm-forming mutant. It is known that CsrA activity is antagonized by two small RNAs, CsrB and CsrC, whose levels are dramatically decreased in a *csrA* mutant, because of an autoregulatory loop in which CsrA production leads to *csrB* transcription activation. Thus, we performed RT-PCR experiments on *csrB* and *csrC* transcripts in order to verify if their expression was reduced in our mutant strain. As shown in Fig. 7, values indicate that *csrB* and *csrC* are highly downregulated (about 150 fold) in PHL1228 strain compared to EB12 (like in a *csrA* mutant). Since our data strongly suggested that the CsrA protein is not active in PHL1228, we sequenced the *csrA* gene from PHL1228, which, surprisingly, carries a wt *csrA* allele. Thus, PHL1228 can be defined as a "*csrA*-like mutant", i.e it is mutated in a yet unidentified gene affecting either CsrA protein activity or *csrA* stability. To test if the mutation might affect the expression of the *csrA* gene, we measured *csrA* transcript levels in the EB12 and PHL1228 strains, but, again, no difference was observed.

We also tried to restore the EB12 phenotype overexpressing *csrA* gene in PHL1228 strain. Interestingly, while in EB12 we succeeded in overexpressing *csrA* (about 13000-fold as measured by RT-PCR experiments), in PHL1228 *csrA* gene expression levels were always very low (about 30-fold, data not shown).
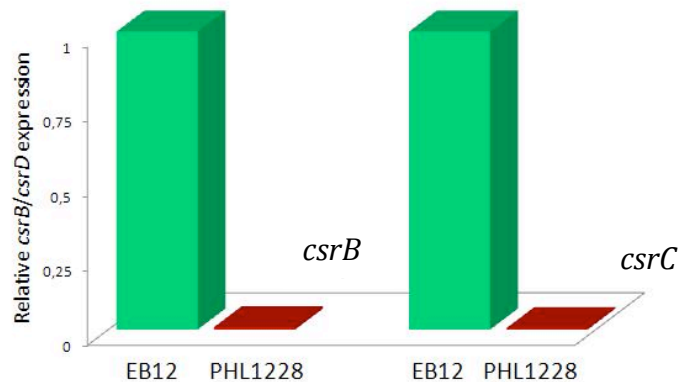
Figure 7. Relative expression levels of the *csrB/csrD* genes in strains EB12 and PHL1228, as measured by Real-Time PCR.

Iron regulation in PHL1228. In addition to increased expression of the *pga* operon, the results of the gene array experiment showed that several iron-dependent genes, such as *fepA*, are also up-regulated in PHL1228. FepA is a protein involved with transport of enterobactin-iron across the outer membrane. In *Yersinia pestis*, the *hms* genes belong to the so-called haemin storage system. Haemin binding and storage was first thought to constitute an uptake or storage system for iron, but *hms* expression regulation by iron is still not totally clear for *Yersinia pestis* (Lillard *et al.*, 1999; Perry *et al.*, 2004; Podladchikova and Rykova, 2006). Thus, it is conceivable that *pga* and iron uptake genes might be co-regulated. We tested whether in PHL1228 *fepA* transcript was regulated, as well as *pgaA*, at post-transcriptional level. Luciferase assay with UTR-deleted construct (data not shown) showed that *fepA* regulation occurs at the UTR level, and transcript decay experiment indicated a stabilization of *fepA* mRNA in the mutant strain (Fig. 8). So, in PHL1228, iron regulation seems to be connected to *pga* expression, possibly via CsrA.

Figure 8. Decay of *fepA* mRNA as determined by Real Time PCR. Blue line, EB12; green line, PHL1228. The dotted horizontal line represents the 50% value; the dotted vertical line shows the time corresponding to the mRNA half-life in EB12. Values are from three different experiments with very similar results.
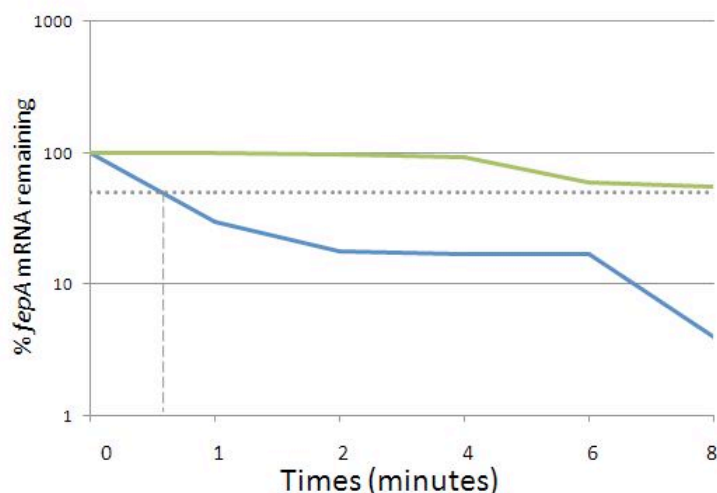
We tested the effect of iron concentration on *pgaABCD* expression (Fig. 9A); depletion of iron from the growth medium obtained through the addition of 2,2'-dipyridyl, a chelating ligand, resulted in a 2-fold increase of *pgaABCD* expression, while growth in the presence of excess of iron had no or little effect. Thus, lack of iron seems to modulate *pgaABCD* activity. Effects of iron concentration were much more dramatic on biofilm formation by PHL1228. Cells growing in M9sup in the presence of 50 µM $FeSO_4$ showed a 50% reduction in biofilm formation compared to cells grown with no added iron. In contrast, cells growing under iron depletion, although impaired in growth (μ_{max}= ca. 50%, data not shown), showed an increase in biofilm formation (Fig. 9B). Microarray experiments in a *csrA* mutant strain of *Salmonella typhimurium* showed an increase in expression of a *fur*-regulated iron transporter (*sitABCD;* Lawhon *et al.*, 2003). Interestingly, co-purification experiments with CsrA protein (in which CsrA was His-tagged and overexpressed in EB12 and PHL1228 strains) showed the presence of 4 peptides corresponding to the Fur protein co-eluted with CsrA from PHL1228 (data not shown). Thus, iron dependence of *E.coli pgaABCD* expression and co-purification results might implicate involvement of the Fur protein in PHL1228; however, no differential expression of either *fur* was detected in gene array or RT-PCR experiments, nor did sequencing of the *fur* gene reveal any mutation (data not shown). Nevertheless, we decided to

analyze the expression levels of other iron-dependent genes, like *tonB*, *fecA* and *entD*. TonB is a cytoplasmic membrane protein, which provides the energy source required for the import of iron-siderophore complexes and vitamin B12 across the outer membrane (Letain, 1997); FecA is the TonB energy transducing system-dependent ferric citrate uptake receptor, while EntD is a component of the enterobactin synthase multienzyme complex. Real-Time PCR experiments (data not shown) highlighted a global deregulation of iron-uptake pathway in PHL1228. The biological meaning of these data and the possible binding CsrA-FuR are yet under investigation.
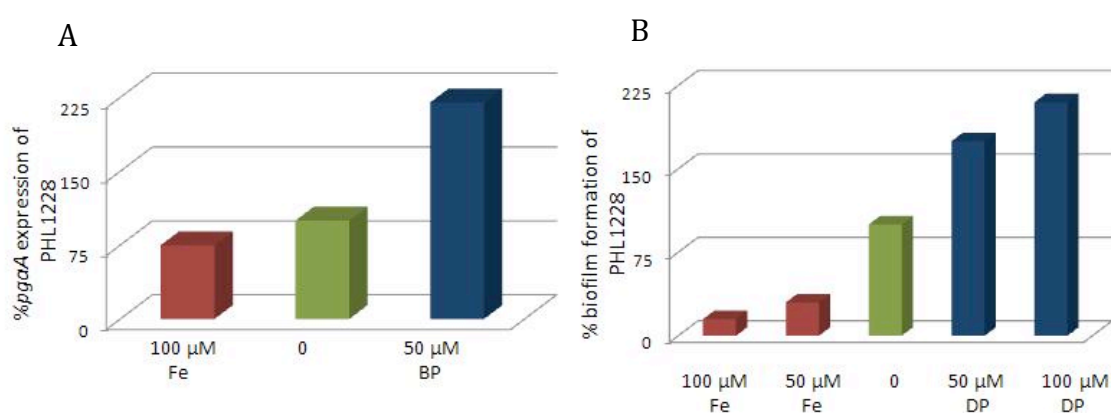
Figure 9. **A)** Luciferase reporter experiments testing the activity of the *pgaA* promoter using PHL1228 cells grown under different conditions: supplemented with $FeSO_4$ (red bars), no addition (green bars), or in presence of the chelator 2,2'-Dipyridyl (DP, blue bars). Values are the average of two independent experiments with very similar results. **B)** Surface adhesion on polystyrene microtiter plates of PHL1228 cells grown in different conditions (as described for panel A).

DISCUSSION

Most laboratory strains of *Escherichia coli* are unable to attach efficiently to solid surfaces and to form biofilm, although they possess the genes encoding for biofilm determinants. However, spontaneous mutations can restore the expression of silenced genes and induce a biofilm-forming phenotype. Curli fibers are an important biofilm determinant in environmental and in pathogenic strains of *E. coli*, and in *Salmonella*. In this report we selected for mutations resulting in the activation of biofilm determinants other than curli: to this aim, we used the EB12 strain, a derivative of MG1655 in which the *csgA* gene,

encoding the main subunit of the curli subunit, had been inactivated. We were able to isolate from a continuous culture of EB12 a spontaneous mutant, the PHL1228 strain, which displays an adherent phenotype (Fig. 1). We expected the mutation resulting in the biofilm phenotype of PHL1228 to positively affect expression of genes encoding biofilm determinants. Thus, we compared gene expression at the whole genomic level in EB12 and PHL1228 mutant derivative. Through this genomic approach we found a high number of differentially expressed genes, consistent with a mutation (or mutations) in regulatory genes. Several biofilm-related genes were found to be differentially expressed in PHL1228, such as *glgS*, encoding the glycogen biosynthesis protein (Beloin *et al.*, 2004; Schembri *et al.*, 2003b). Glycogen synthesis provides glucose polymers used in the synthesis of extracellular substances, and is a biofilm determinant in *Salmonella enteritis* (Bonafonte *et al.*, 2000); however, changes in the expression levels of these proteins might be a result of PHL1228 growth physiology rather than the main determinant for biofilm formation in this strain. Interestingly, the *pgaABCD* locus was among the genes more highly up-regulated in PHL1228 (Fig. 2). Inactivation of the *pgaA* gene resulted in loss of the PHL1228 biofilm-forming phenotype, thus strongly suggesting that increased expression of the *pgaABCD* operon is the main biofilm determinant in PHL1228 (Fig. 3). The *pgaABCD* locus encodes for proteins responsible for poly-*N*-acetylglucosammine biosynthesis (PNAG), an extracellular polysaccharide involved in biofilm formation and adhesion in several strains, such as *Y. pestis*, *S. aureus* and *E. coli*. In *E. coli* strains, *pgaABCD* operon is regulated at post-transcriptional level by CsrA, a small protein that binds to the leader of target mRNAs, inhibiting their translation and altering their stability. From the literature it is known that CsrA binds to six sites on *pgaA* transcript (Wang *et al.*, 2004). Thus, we tested if *pgaA* expression could be regulated at the translation level in the PHL1228 strain as well. Luciferase assays with ΔUTR constructs suggest that up-regulation of *pgaA* transcript takes indeed place at post-transcriptional level in PHL1228 (Fig. 5). Transcript decay experiments confirmed that *pgaA* mRNA is more stable in the PHL1228 strain compared to its parental strain (Fig. 6). Moreover, Real-Time PCR experiments showed a strong decrease (more than 100-fold) in *csrB* and *csrC* transcripts, as occurs in a *csrA* mutant strain (Fig. 7); expression of CsrB and CsrC is indeed positively regulated by CsrA, in an autoregulatory loop, via UvrY or independently of it.

Although PHL1228 behaviour (biofilm formation, cell aggregation and gene expression pattern) would be in line with a mutation in the *csrA* gene, no mutation in the *csrA* gene from PHL1228 could be detected. Thus, PHL1228 seems to be a "*csrA*-like mutant", i.e. it is mutated in a yet unidentified gene affecting either CsrA protein activity or *csrA* stability.

In addition to the results concerning *pgaA* stability, gene array results pointed to an increase in the PHL1228 strain of *fepA*, a gene involved in iron uptake. Luciferase assays with UTR-deleted constructs showed that *fepA* regulation (like *pgaA*) occurs at the UTR level, and transcript decay experiments showed a stabilization of *fepA* mRNA in the mutant strain (Fig. 8). Starting from these data we analyzed other iron-dependent genes, like *tonB*, *fecA*, *entD*, *fhuA* and RT-PCR experiments showed that there is a global deregulation of iron-uptake pathway (data not shown). We investigated if this deregulation might be due to a different expression of the Ferric uptake regulator (*fur*) gene, but no differential expression of *fur* was detected in gene array or RT-PCR experiments (data not shown). Interestingly, Fur peptides co-eluted with purified CsrA protein overexpressed in PHL1228 strain but not in EB12 strain (data not shown), suggesting a possible interaction of these two proteins in our biofilm-forming mutant. Iron-dependent regulation of biofilm formation varies by bacterial species, and the exact regulatory pathways that control iron-dependent biofilm formation are often unknown or only partially characterized. Iron is a key nutrient that has been shown to regulate biofilm formation in multiple bacterial species. In some species, such as *Legionella pneumophila*, *Staphylococcus aureus*, and *Streptococcus mutans*, iron limitation induces biofilm formation (Berlutti *et al.*, 2004; Hindre *et al.*, 2008; Johnson *et al.*, 2005). In contrast, iron limitation inhibits biofilm formation in other species such as *Vibrio cholera* and *Xylella fastidiosa* (Cursino *et al.*, 2009; Koh and Toney, 2005; Banin *et al.*, 2005; Mey *et al.*, 2005). However, iron regulation of biofilm formation can be quite complex even within the same species. The complicated relationship between iron availability and biofilm formation has been most well studied in the opportunistic pathogen, *Pseudomonas aeruginosa*. In *P. aeruginosa*, iron limitation can reduce biofilm formation by blocking early steps in microcolony formation (Banin *et al.*, 2005). The iron-chelation activity of human lactoferrin can also diminish *P. aeruginosa* biofilm formation, and it has been suggested that this may play a role in limiting

infection. This suggests that up-regulation of iron-dependent genes in PHL1228 is correlated with *pgaABCD* expression and consequent biofilm formation and that adherent phenotype depends on concerted production of different determinants, whose expression is also affected by iron concentration. Characterization of the mutation responsible for the PHL1228 biofilm phenotype is currently being undertaken; an attractive approach could be to sequence the whole EB12 and PHL1228 genome to identify the mutation responsible for the PNAG-producing phenotype of PHL1228.

METHODS

Bacterial strains and growth conditions. Bacterial strains used in this work are listed in Table 1. When not otherwise stated, bacteria were grown in M9Glu/sup (M9 inorganic salts (Smith and Levine, 1964), 5 g/L glucose, 0.25 g/L Peptone, 0.125 g/L Yeast Extract). When needed, antibiotics were used at the following concentrations: ampicillin, 100 µg/ml; chloramphenicol, 50 µg/ml; kanamycin 50 µg/ml; rifampicin, 100 µg/ml.

Biofilm formation assays. Biofilm formation in microtiter plates was determined essentially as described (Dorel *et al.*, 1999). Bacterial cultures were grown overnight in M9Glu/sup at 30°C in polystyrene microtiter plates (0.2 ml); cell density of the culture was determined spectrophotometrically at 600nm (OD_{600nm}). Cells attached to the microtiter plates were washed gently with water and stained for 20 min with 1% crystal violet (CV), thoroughly washed with water and dried. For semi-quantitative determination of biofilms, CV-stained cells were resuspended in 0.2 ml of 95% ethanol by vigorous pipetting. The OD_{600nm} of each sample was determined and normalized to the OD_{600nm} of the corresponding liquid cultures (Adhesion units). Sensitivity of biofilms to treatment with the PNAG degrading enzyme Dispersin B (Kaplan *et al.*, 2004; purchased from Kane Biotech Inc., Winnipeg, Canada) was performed by adding 20 µg/ml of the enzyme to the growth medium.

Plasmid construction. Plasmids used in this work are listed in Table 1.

Luciferase assay. Luciferase assays were performed as described below, using the vector pJAMA8 (Jaspers *et al.*, 2000), which carries promoterless *luxAB* genes from *Vibrio harveyi*. 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 deleted (ΔUTR, ranging from -116 to +23 nucleotides relative to the *pgaABCD* mRNA start site) were amplified from the chromosomal DNA using primers including *SphI* and the *XbaI* restriction sites and cloned into the multiple cloning site of pJAMA8 to obtain pPgaA$_{WT}$ and pPgaA$_{ΔUTR}$, respectively. Bacterial strains containing the different reporter plasmids were grown overnight. The samples were adjusted to an OD$_{600}$ of 0.05–0.1 in PBS buffer. 20 µl of this solution was tested for luciferase activity by adding 200 ml PBS containing n-decanal to a final concentration of 2 nM. Measurement of relative light units (RLU) was conducted by a 2 s pre-measurement delay followed by a 3 s measurement after addition of the substrate in a MicroLumat LB 96 P luminometer (Berthold Technologies). Results are expressed as RLU per OD$_{600}$ of the tested bacterial samples.

RNA isolation and gene array assay. Total RNA from *E. coli* cells grown overnight to stationary phase in M9Glu/sup at 30°C was isolated with RNeasy mini kit (Qiagen) including an on-column DNase I treatment. RNA samples were quantified using a spectrophotometer (260 nm) and checked by gel electrophoresis. For gene array experiments, fluorescently labeled cDNA from 25 µg total RNA was produced using the CyScribe First-Strand cDNA Labeling kit (Amersham biosciences) incorporating Cy3- or Cy5-dCTP respectively. Labeled cDNAs were pooled and purified with the Minielute PCR purification kit (Quiagen) and concentrated with a Microcon-30 (Millipore) prior to the addition of the hybridization buffer. For our experiments we used the *E. coli* K-12 V2 Array (MWG) containing 4286 genes (http://www.mwg-biotech.com/) according to the manufacturer's instructions.

Gene array data analaysis. Microarray slides were scanned using the Affimetrix 428^{TM} Array Scanner (High Wycombe, UK). Spots and corresponding background signals of obtained sixteen-bit TIFF images were quantified using the Affimetrix Jaguar^{TM} software version 2. Subsequent data analysis was performed using the program GeneSpring 4.1 from Silicon Genetics (Redwood City, USA). Induction factors (PHL1228 compared to control) were calculated using the Cy3 and Cy5 signal intensities of each spot. Spots with control signals below a value of 10 were excluded from the analysis and the minimal treatment to control ratio was set to 0.01. Normalization was performed using the 50th percentile distribution of remaining spots after background correction. Only PHL1228 signals values higher than 400 for up-regulated and EB12 signals for down-regulated genes respectively were considered. Finally, genes having an average Induction Factor (IF) of at least 4.5, and a signal to control ratio in both of the two experiments of more than 2.5, were chosen.

Gene expression studies. Real-Time PCR for determination of relative expression levels was performed on overnight cultures grown in M9Glu/sup medium at 30° C. Primers for Real- Time PCR are listed in Table 2. RNA extraction and further Reverse Transcription and cDNA amplification steps were performed as described (Gualdi *et al.*, 2007), using 16S RNA as reference gene. mRNA stability was measured by Real-Time PCR experiments in the presence of rifampicin as described (Wang *et al.*, 2005).

Outer membrane proteins. Isolation of outer membrane proteins (OMPs) was carried out using the Sarkosyl extraction method as described in Landini & Zehnder, 2002. 40 µg of OMP were analyzed by sodium dodecyl sulfate-polyacrilamide gel electrophoresis (SDS-PAGE). Protein bands of interest were extracted from the gel and identified by mass spectroscopy analysis of trypsin cleavage products as previously described by Chen *et al.*, 2000.

Electron microscopy. Bacterial cells from overnight liquid cultures were fixed with 2.5% glutaraldehyde and prepared for transmission electron microscopy as described in Kessi *et al.*, 1999.

Table 1. Strains and plasmids used in this work.

E. coli strains	Relevant characteristics	Reference
EB12	MG1655 *csgA*::*uidA*-Kan; malT54::Tn10	This study
LT106	PHL1228 *pgaA*::cat	This study
MG1655	K-12, F⁻ λ⁻ rph-1	Blatter *et al.*, 1997
PHL1228	EB12, biofilm-forming mutant	This study

Plasmids

pJAMA8	Control vector for luciferase assays, ampicillin resistance	Jaspers *et al.*, 2000
pPgaA$_{WT}$	*pgaA* promoter and regulatory region (-116 to +233 relative to transcription start site) cloned into the *SphI*/*XbaI* sites of pJAMA8	This study
pPgaA$_{\Delta UTR}$	*pgaA*$_{\Delta UTR}$ (-116 to + 23 relative to transcription start site) cloned into the *SphI*/*XbaI* sites of pJAMA8	This study

Table 2. Primers used in this work.

Primers	Sequence	Utilization
pPgaA-delUTR _for	GCATGCAACAATTAAATCCGTGAGTGCCG	*pgaA* promoter cloning
pPgaA-delUTR_rev	TCTAGAATCTTCAGGAATACGGCATAAAT	*pgaA* promoter cloning
pPgaA_wt_for	AGCATGCCTCAAATAGTCTTTTTCCAT	*pgaA* promoter cloning
pPgaA_wt_rev	ATCTAGATACATCCTGTATTACTCCATG	*pgaA* promoter cloning
16s_for	TGTCGTCAGCTCGTGTCGTGA	qRT-PCR
16s_rev	ATCCCCACCTTCCTCCGGT	qRT-PCR
pgaA_RT_for	CCGCTACCGTCATCAGCAATT	qRT-PCR
pgaA_RT_rev	AGCGCCTTTTGCCACAGTGT	qRT-PCR
pFepA_for	CTGCATGCCCATGTTTCGACTGCCACCA	*fepA* promoter cloning
pFepA_wt_rev	CTTCTAGACAAGGCCAGGGAATGAATCTTC	*fepA* promoter cloning
pFepA-delUTR_rev	TTTCTAGACGCGCCATTACGCTATTGC	*fepA* promoter cloning
pgaA_cat_for	ATACAGAGAGAGATTTTGGCAATACATGGAGT AATACAGGTACCTGTGACGGAAGATCAC	*pgaA* inactivation
pgaA_cat_rev	ATCAGGAGATATTTATTTCCATTACGTAACATA TTTATCCGGGCACCAATAACTGCCTTA	*pgaA* inactivation
pgaA_for	TGGACACTCTGCTCATCATTT	Mutant verification
fepA_RT_for	ATTCCCTGGCCTTGTTGGTCA	qRT-PCR
fepA_RT_rev	GGCGGTAACGACAATAGTATCG	qRT-PCR
csrB_RT_for	GGGAGTCAGACAACGAAGTG	qRT-PCR
csrB_RT_rev	CTGACCGGTTCTCATTCTCC	qRT-PCR
csrC_RT_for	TAGAGCGAGGACGCTAACAG	qRT-PCR
csrC_RT_rev	AACGGGTCTTACAATCCTTGCAG	qRT-PCR

CONCLUDING REMARKS

CONCLUDING REMARKS

In this PhD thesis work I investigated the expression modulation of the major adhesion factors in *Escherichia coli*; in particular I focused on the role of GGDEF and EAL proteins, on their modulation in *E. coli* biofilm formation in response to environmental signals and on regulation of curli fibers, cellulose and poly-*N*-acetylglucosamine (PNAG), the most important biofilm determinants in *E. coli*.

E. coli is an Enterobacterium, normally living inside the mammalian gut, at temperature of 37° C and in relatively nutrient-rich environment. Once outside the host, bacteria usually face much lower temperatures (< 30°C) and a nutrient-limiting environment. The biofilm determinants studied in this thesis are all expressed in response to environmental conditions such as low temperature, low osmolarity and starvation, suggesting that *E. coli* bacteria switch to a biofilm mode of growth as part of their adaptation to the natural environment. In response to reduction in growth rates, *E. coli* seems to canalize its energy consumption into production of extracellular features such as curli or exopolysaccharides. Biofilms can be thus considered as a "resistance form" of growth able to withstand stress conditions more efficiently than cells living in a planktonic mode of growth.

The CsgD protein is the master regulator of *E. coli* biofilm formation. It is a transcriptional factor necessary for curli genes transcription and, through the AdrA protein, for cellulose biosynthesis. Gene regulation by CsgD is tightly connected to production and sensing of cyclic di-GMP, a bacterial second messenger involved in various cellular processes, including biosynthesis of extracellular polysaccharides (Simm *et al.*, 2004), biofilm formation (Hickman et al., 2005), and virulence (Pratt *et al.*, 2007; Tischler and Camilli, 2005), as well as morphological and physiological differentiation (Paul *et al.*, 2004). The CsgD-dependent *adrA* gene, involved in cellulose biosynthesis (Zogaj *et al.*, 2001), encodes a cyclic di-GMP synthase (Simm *et al.*, 2004). CsgD can also activate *yoaD*, whose gene product is a cyclic di-GMP phosphodiesterase, suggesting that CsgD is directly involved in feedback regulation of cyclic di-GMP intracellular levels and of cellulose biosynthesis (Brombacher *et al.*, 2006).

CsgD is also able to activate the *iraP* gene: IraP acts as a stabilization factor for the σ^s protein, an alternative sigma factor of RNA polymerase which directs transcription of genes involved in adaptation to slow growth and to cellular stresses. Here I showed that CsgD transcription activation of the *iraP* gene does result in a significant increase of σ^s intracellular concentration by positively affecting σ^s protein stability, thus leading to altered expression of σ^s-dependent genes. CsgD-mediated increase of σ^s cellular concentrations via the *iraP* gene would trigger an autoactivation loop leading to an increased production of CsgD-dependent adhesion determinants such as curli fibers and cellulose. This autoregulatory circuitry might be further fueled by σ^s-dependent induction of genes encoding di-guanylate cyclases, i.e., proteins able to synthesize the second messenger di-cyclic- GMP, which, in turn, can positively affect *csg* gene expression (Kader *et al.*, 2006; Weber *et al.*, 2002).

The *yddV-dos* operon is the most expressed among c-di-GMP-related genes showing dependence on σ^s (Weber *et al.*, 2006; Sommerfeldt *et al.*, 2009). It encodes, respectively, a protein with DGC activity and a PDE that can degrade c-di- GMP to pGpG. Both Dos and YddV are heme-binding oxygen sensors, and interact to form a stable protein complex (Tuckerman *et al.*, 2009). Although it has been reported that YddV overexpression can stimulate biofilm formation (Mendez-Ortiz *et al.*, 2006), the targets of *yddV*-dependent biofilm induction had not yet been identified. Here I showed that YddV acts modulating curli and PNAG expression. Control of curli production by *yddV-dos* takes place at the level of transcription regulation of the *csgBAC* operon, encoding curli structural subunits, and is mediated by the DGC and PDE activities of YddV and Dos. In contrast, the YddV–Dos protein complex does not strongly influence *csgDEFG* expression, nor does it affect the expression of the CsgD-dependent *adrA* gene, encoding a positive effector for cellulose biosynthesis. Regarding PNAG production, we showed that YddV is able to prevent degradation of *pgaABCD* transcript in the MG1655*csrA* background, thus suggesting that a DGC might regulate gene expression by affecting mRNA stability in *E. coli*. YddV regulation of *pgaABCD* operon in a wild type contest is still controversial: *pgaABCD* genes are expressed at low levels in MG1655 (the standard laboratory strain of *E. coli*) and their mRNA half-life is lower than two minutes regardless of the growth conditions tested; thus, possible effects of

yddV inactivation on destabilization of the *pga* transcript are not easy to evaluate in the wt contest.

In the last part of my thesis I tried to characterize a biofilm-forming mutant of *E. coli*, able to express *pgaABCD* genes at high levels. Even if initial data suggested that a mutation in the *csrA* gene could be responsible for *pga* mRNA stabilization in this mutant, actual the mutation leading to the adhesive phenotype and to PNAG production is outside the *csrA* gene and is still unknown. Moreover my data suggest a connection between *pga* expression and iron regulation in *E. coli* strains: it is conceivable that *pgaABCD* expression and consequent biofilm formation and the adherent phenotype depends on concerted production of different determinants, whose expression is also affected by iron concentration.

Thus, my research highlighted that biofilm production is the result of coordinated expression of different adhesion determinants, whose regulation is complex and not fully understood. In particular, the precise extent and the molecular mechanism of c-di-GMP adhesion factors regulation remains to be largely identified and represents an exciting challenge for future research in the biofilm field.

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APPENDIX