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Role of genes belonging to metabolic pathways (sulfate assimilation and pyrimidine biosynthesis) in the production of extracellular structures in *Escherichia coli*.

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Contents

Abstract

Most bacteria are able to grow as single cells or organized in microbial communities attached to solid surfaces, known as biofilms. Growth as a biofilm confers bacterial cells different advantages, such as increased resistance to environmental stresses, antimicrobial agents and it also protects the bacteria from host immune system. During the transition from planktonic cells to biofilm bacteria undergo a genome-wide reprogramming of gene expression that leads to the production of specific biofilm determinants and to deep physiological changes. This process requires the consumption of a large amount of energy and therefore the metabolic state of the cell plays a central role in regulating the biosynthesis of adhesion structures. Indeed, several metabolic pathways and metabolites have been shown to directly control the production of biofilm determinants.

During my Ph.D, I have tackled the problem of how different metabolic pathways can regulate the production of extracellular structures involved in biofilm formation in *Escherichia coli*. In particular, we showed that curli and cellulose, the two main adhesion factors in *E. coli*, are affected by nucleotide biosynthetic pathways: transcription of curli encoding operons responds to pyrimidine nucleotide availability, while cellulose production is triggered by exogenous uracil in the absence of active *de novo* UMP biosynthesis. Furthermore, we showed that curli production is also hindered by the reduction of purine nucleotides. Since curli and cellulose are pivotal for biofilm formation, nucleotide biosynthesis could be a good candidate for drugs endowed with anti-biofilm activity. Indeed, we showed that the drug azathioprine, which inhibits nucleotides biosynthesis, could prevent biofilm formation in clinical isolates of *E. coli* through the reduction of the nucleotide pools available for the synthesis of the second messenger c-di-GMP.

Nucleotide biosynthesis is not the only metabolic process that influences the production of biofilm determinants in *E. coli*. We showed that curli fibres production is also affected by yet another biosynthetic pathway, namely, sulphate reduction for cysteine/methionine biosynthesis. Our data suggest that accumulation of pathway intermediate phosphoadenosine 5'-phosphosulfate (PAPS) affect the production of curli fibres and of other extracellular structures.

Therefore my results strongly support the idea that metabolic fluxes of different essential elements play a crucial role in controlling cell surface reorganization. Accumulation of intermediate metabolites is instrumental in relaying to the bacterial cell conditions of lack or abundance of a given element, triggering the adequate responses. The molecular mechanisms involved in this process seem to be extremely complex and probably involve gene regulation control at transcription initiation and RNA and protein stability.

PART I

1 Bacterial biofilms

In natural environments, bacteria face complex and dynamic conditions. Bacteria often live in sub-optimal niches, where they are subjected to limited availability of nutrients, desiccation, low pH and predation. In response to unfavourable situations bacteria can switch from a single-cell life style to a multicellular community form, known as biofilm (Karatan and Watnick, 2009). Biofilms are characterized by tightly associated cells embedded in a matrix composed by extracellular polysaccharides (EPSs), proteins and, depending on the bacterial species, extracellular DNA (eDNA) (O'Toole et al., 2000; Whitchurch et al., 2002; Kolter and Greenberg, 2006).

Cells growing in biofilms greatly differ from planktonic bacteria (Costerton et al., 1995); during the transition from single cell to multicellular community they are subjected to radical morphological and biochemical changes (Beloin et al., 2004; Ren et al., 2004; Schembri et al., 2003).

The tight association in a biofilm fosters the exchange of metabolic products and diffusible signals between bacteria, therefore influencing the physiological state of cellular sub-populations and leading to a rudimental functional differentiation, resembling a simple multicellular organism (Caldwell, 2002; Shapiro, 1998; Costerton et al., 1995). Moreover, biofilm structure and lack of motility promote horizontal DNA exchange (Ghigo, 2001), further increasing the genetic variability among the bacterial community (Boles et al., 2004), and its capability to adapt to environmental changes (Kolter and Greenberg, 2006).

1.1 Biofilms role in the industry and human health

Biofilms are ubiquitous (Costerton et al., 1995), they can be found on different surfaces, either biotic or abiotic, and have a tremendous impact on industry and on human health (Costerton et al., 1987).

Bacterial biofilms have both positive and negative comebacks for human activities. Indeed, they are extensively used in water treatment plants and during bioremediation processes to improve and facilitate removal of toxic compounds from waste waters and contaminated soils (Nicolella, 2000; Dash et al., 2013). On the contrary, bacterial contaminations can hamper industrial processes (Dourou et al., 2011; Torres et al., 2011) and biofilms adhesion to metal surfaces promotes corrosion, resulting in important economical losses (Costerton et al., 1995). Biofilm removal is

carried out using either biocides or mechanical methods (*i.e.* grinding, wash-out with high-pressure water), but the complete and efficient eradication is often difficult (Bruellhoff et al., 2010).

Bacteria also colonize medical devices (from urinary catheters to contact lens) usually compromising not only the correct functionality of the device but also the human health (Donlan, 2011). Indeed, biofilm formation is often associated with human diseases, and represents an important step in the chronicization of several different pathologies. According to the Centre for Disease Control (CDC), 65% of all infections in the US are caused by microbial biofilms, while the National Institutes of Health estimates that this percentage is roughly 80%. Sources of infection include commensal microbes that live on human body surface, such as staphylococci, and *Pseudomonas aeruginosa* (Joo and Otto, 2012). Biofilm-associated diseases are often more difficult to treat and require a considerable amount of time and higher antibiotics doses before they can be completely eradicated (Hoyle and Costerton, 1991; Finlay and Falkow, 1997; Donlan and Costerton, 2002; Gilbert et al., 2002). Eradication problems arise because cells living in a biofilm are less sensitive to antimicrobial agents compared to planktonic bacteria (Costerton et al., 1995; Mah et al., 2003; Martínez and Rojo, 2011). Three different mechanisms have been proposed to explain the reduced susceptibility to antibiotics shown by bacterial biofilms. The first is the barrier properties of the extracellular matrix that surrounds bacteria. Antimicrobial agents are bound and neutralized by EPS or are diluted to sublethal concentration before reaching the innermost portion of the colony (Hall-Stoodley et al., 2004). The second mechanism involves the environmental and growth condition of biofilm organisms. Although many antibiotics freely penetrate the EPS, the presence of starved and stationary phase bacteria seems to play a pivotal role in bacteria survival to antimicrobial (Spoering and Lewis, 2001; Anderl et al., 2003; Walters et al., 2003). The last mechanism proposes the existence inside biofilms of subpopulations of regular cells that are highly tolerant to antibiotics, which are known as persisters (Spoering and Lewis, 2001; Percival et al., 2011). The contribution of each of these mechanism varies within each environment and bacterial species, offering a different degree of protection to the cells (Hall-Stoodley et al., 2004).

Though most of research focused their attention on understanding and preventing the negative impact of biofilms on human health, bacteria communities growing in large intestine attached to digestive wastes play an important role in digestive processes, gut physiology, and metabolism (Macfarlane and Macfarlane, 2006). Thus, it is clear that during biofilm treatment it is important to achieve a balance between preventing microbial communities associated with disease and maintaining or promoting beneficial biofilms.

1.2 Biofilm development

Research has supported the view that most, if not all, bacteria, are able to form biofilm and that probably they spend a considerable part of their life as part of a biofilm community (Costerton et al., 1995; Stoodley et al., 2002). Thus, each bacterial consortia presents unique characteristics originating from the combination of bacterial species and extracellular condition in which it develops (Bridier et al., 2010).

Currently, the most accepted model describing biofilm formation is the so called developmental model, in which biofilm is seen as a complex multi-step process, where five different stages can be identified (O'Toole et al., 2000) (see Figure 1).

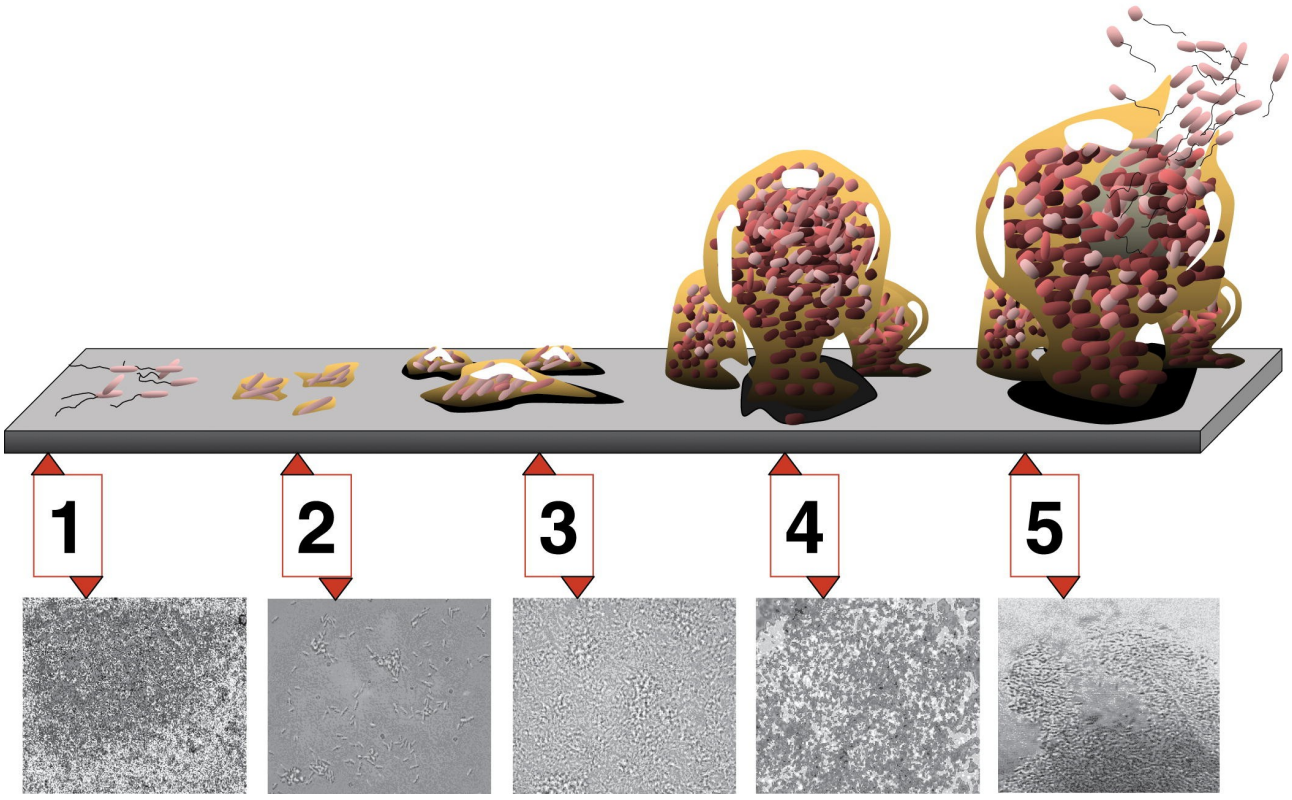

Figure 1. Schematic representation of biofilm developmental model. From Monroe, 2007.

In the first step, bacteria undergo an initial reversible adhesion to the surface, which is mostly controlled by physiochemical properties such as Van der Waals interactions, electrical charge and hydrophobicity of both bacterial cells and surfaces (Jucker et al., 1996; Van Loosdrecht et al., 1990) (Figure 1, stage 1). Upon sensing the contact with the surface, bacteria start a specific genetic program, leading to a further development of biofilm (Sauer and Camper, 2001). In the presence of opportune conditions, adhesion become irreversible and cells start to duplicate forming a monolayer called microcolony (Figure 1, stage 2). Cell to cell interaction become stronger allowing the

differentiation of a mature biofilm whose three-dimensional structure is determined by the extracellular polymeric substance, which surround the bacterial colony (Figure 1, stages 3 and 4). Extracellular polymeric substance is mainly made up of exopolysaccharides (EPS), proteins, enzymes, and extracellular DNA (Karatan and Watnick, 2009). Biofilm commonly develops to form a vertical structure whose thickness varies within a very wide range (from micrometres to centimetres). The thicker a biofilm, the more likely the creation of different microenvironments characterized by specific physicochemical conditions that can support the growth of heterogeneous bacterial species (Stoodley et al., 2002).

In the last step of its life cycle, biofilm undergo a structural fragmentation with the release of cell-clumps and single cells. Although this process can be influenced by mechanical events, *i.e.* the exposure to high flow, biofilm detachment and desegregation is a cell-driven process carried out in response to different environmental signals, such as nutrient reduction (Sauer et al., 2004; Gjermansen et al., 2005) or the accumulation of oxygen and nitrogen reactive species (Barraud et al., 2006). Thus, biofilm dispersion could represent an effective way for the cells to escape adverse condition generated inside the community. In order to escape from biofilms, bacteria disrupt the extracellular matrix that encases them through the active synthesis of EPS-degrading enzymes (Nijland et al., 2010; Abee et al., 2011).

About ten years after the proposal of the developmental model, it was argued that, while a development model require a specific program to be followed (cascade of modules), as described in other bacterial cell processes, such as *Bacillus* sporulation and *M. xanthus* formation of fruiting bodies, little evidence supports the idea that biofilm formation relies on an independent and dedicated gene network. On the contrary, biofilm-related pathways seem to involve a mosaic of genetic modules that have been co-opted and integrated to facilitate regulation of biofilm formation (Ghigo, 2003; Monds and O'Toole, 2009). Thus, these authors proposed a second model in which individual bacteria respond to their own specific and local environment. This model does not hold onto the idea that the response to an environmental signal follows a biofilm-specific regulatory pathway in each cell. Instead, it proposes that the change in biofilm structure and amount may be due to central metabolic modifications due to the environment conditions (Monds and O'Toole, 2009; Prüß et al., 2010) (Figure 2).

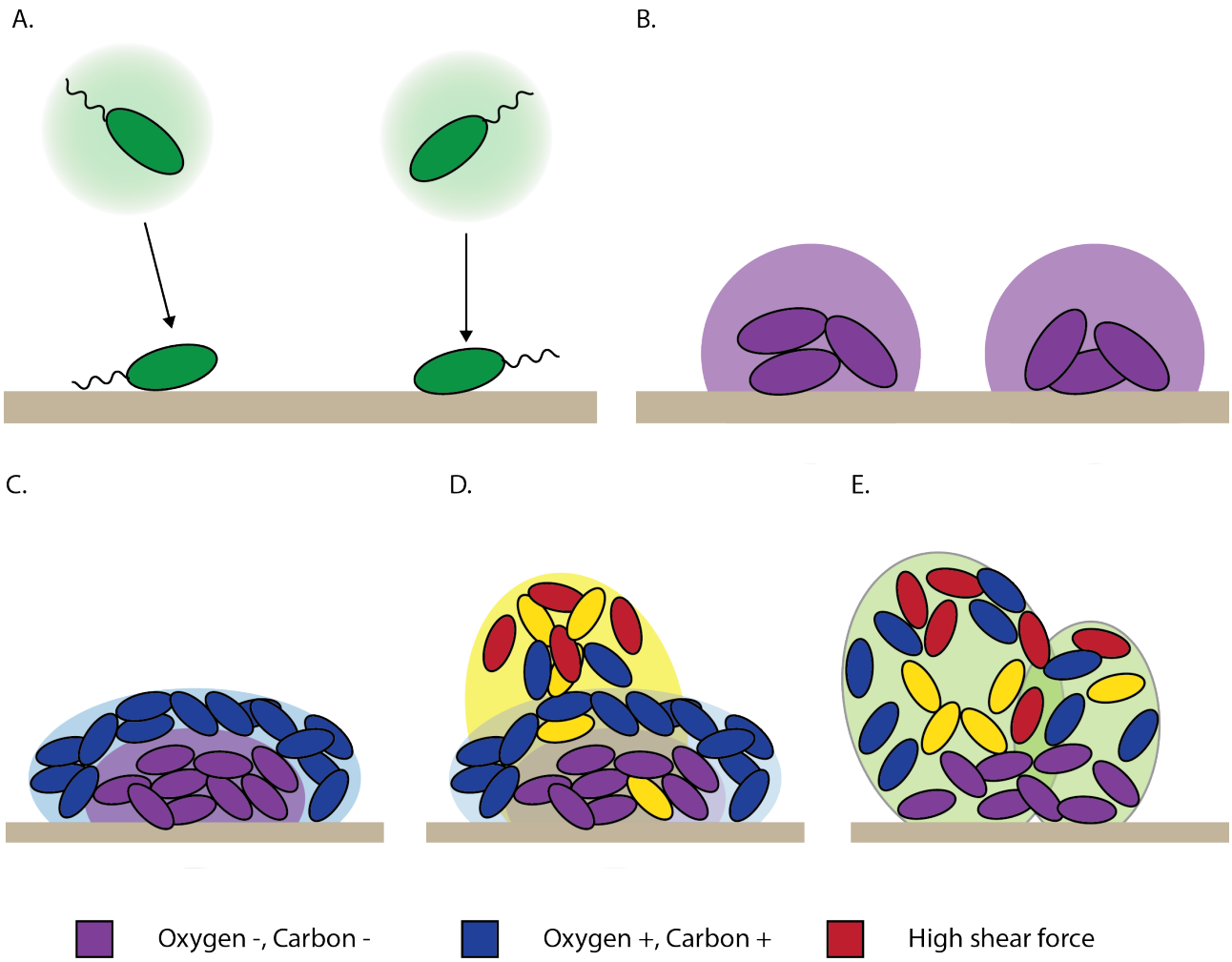

Figure 2. Local ecological adaptation of individuals model. Adapted from Monds and O'Toole, 2009. **A.** In response to signals two cells attach independently to a surface. **B.** Sufficient levels of oxygen and carbon favours cell division. **C.** A microcolony is formed. Metabolism of superficial bacteria restricts diffusion of oxygen and carbon to the bottom cells that need to adapt to the newly created conditions, creating phenotypic heterogeneity within the colony. **D.** Stochastically a portion of cell expresses higher levels of EPS (yellow cells), promoting vertical growth, biofilm maturation and the formation macrocolonies. As the vertical growth proceeds, top cells are subjected to higher shear forces (yellow zone), and adapt to this new local environment, further increasing cellular heterogeneity. **E.** Mature macrocolonies fuse to form a mature biofilm.

While the developmental model proposes a completely deterministic explanation of biofilm formation in which cells undergo a specific expression program, models based on ecological adaptation of individuals suggest that responses to environmental signals are integrated with stochastic interactions with the ever-changing microenvironments inside the same biofilm, ultimately leading to biofilm formation without following a step-by-step program (Monds and O'Toole, 2009). Both models try to describe a complex and broad phenomenon, and it is possible that one model can fit better than the other on a case-by-case basis.

2 Determinants involved in biofilm formation

Despite the model chosen for describing the biofilm formation process, during the transition from planktonic cells to biofilm, bacteria are subjected to substantial modifications to their morphology and biochemistry, mainly due to changes in gene regulation and expression (Beloin et al., 2004; Ren et al., 2004). Several features required for biofilm formation have been described, most of which are surface-exposed or extracellular structures involved in cell-to-cell and cell-to-surface adhesion; the main adhesion determinants are extracellular polysaccharides (Ryder et al., 2007; Vu et al., 2009), pili, flagella (Klausen et al., 2003), membrane proteins (Newell et al., 2009) and extracellular DNA (Whitchurch et al., 2002) (Figure 3).

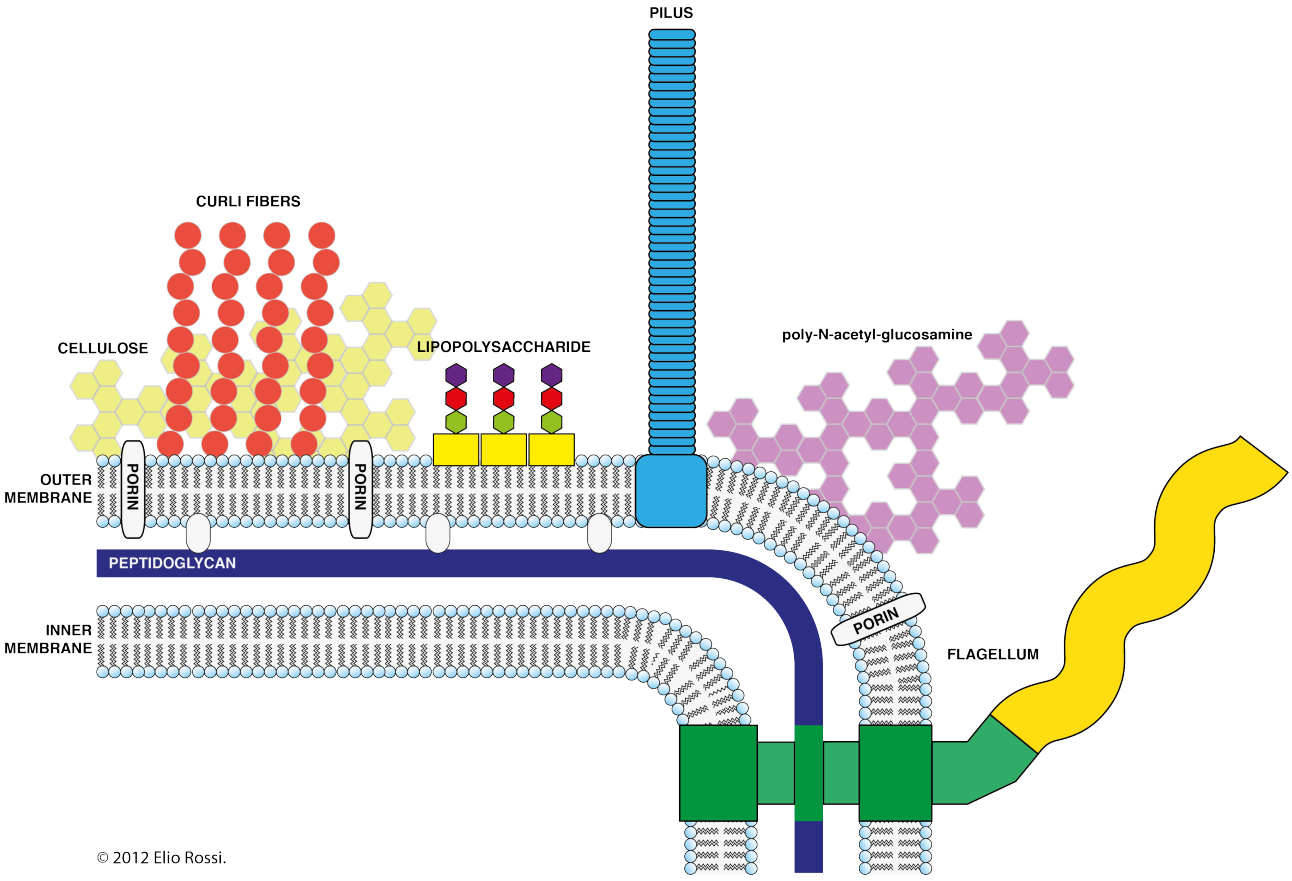

Figure 3. Schematic representation of the main extracellular structures involved in cellular adhesion in the Gram-negative bacterium *Escherichia coli* K-12.

The next sections will present a general overview of these bacterial structures with a particular focus on Gram-negative bacteria (see Table 1, pag. 18-19 for a summary).

2.1 Extracellular polysaccharides (EPS)

Extracellular polysaccharides, or exopolysaccharides (EPS), are long, thin molecular chains with a mass of about 0.5-2.0x10^6 Da, which can associate in a number of different ways to form a very complex network structure (Sutherland, 2001). The role of these polysaccharides includes maintaining structural integrity of cell envelope, preventing cellular desiccation (Whitney and Howell, 2013), as well as promoting the correct shaping and maturation of biofilm (Branda et al., 2005) and contributing to the protection of bacteria from environmental stresses and bactericidals (Hall-Stoodley et al., 2004). Although these polymers constitute the main component of the extracellular matrix (Ryder et al., 2007), the exopolysaccharides do not exist alone but interact with a wide range of other molecular species, including lectins, proteins, lipids, as well as other polysaccharides (Sutherland, 2001). EPS vary greatly in their composition and in their chemical and physical properties; they could be specific to a strain, but a common theme that emerges is that individual strains are often able to synthesize several different exopolymers that characterize the biofilm matrix of a specific *genus* (Branda et al., 2005).

In Gram-negative bacteria, the molecular mechanism by which these biopolymers are assembled and exported from the cell can currently be categorized into three distinct mechanisms; the first is represented by Wzx/Wzy-transporter-dependent pathway, which uses a lipid as an acceptor; examples of this mechanism are the *E. coli* group 1 capsular polysaccharides (CPS) and O-antigen production. The second system, which relies upon ATP-binding cassette (ABC) transporters, assembles the entire polysaccharide on a lipid acceptor and is used, for example, in the production of *E. coli* group 2 CPS and LPS common antigen. The synthase-dependent pathway, a third mechanism of assembly, for which the requirement for a lipid acceptor depends on the polysaccharide, is typical of complex polymers such as alginate, cellulose, acetylated cellulose and poly-*N*-acetylglucosamine (PNAG) (Whitney and Howell, 2013).

Alginate is a high molecular weight acetylated polymer made up of non-repetitive monomers of ß-1,4-linked L-guluronic and D-mannuronic acid and represents the predominant extracellular matrix component of *Pseudomonas aeruginosa* mucoid colonies isolated from patients affected by cystic fibrosis (Deretic et al., 1990); although alginate is an important biofilm determinant in this bacterium (Hentzer et al., 2001), non-mucoid strains and strains lacking alginate-synthase complex are still able to form biofilm and maintain the colony three-dimensional structure (Friedman and Kolter, 2003; Wozniak et al., 2003). Indeed, *P. aeruginosa* is capable to produce two other carbohydrate-rich polymers that make significant contribution to biofilm structure. PelA-G proteins

mediate the synthesis of PEL, a glucose-rich polymer (Matsukawa and Greenberg, 2004), whereas PslA-O proteins mediate the synthesis of a mannose-rich polymer called PSL (Jackson et al., 2004).
In addition to the three exopolysaccharides already described, a subset of *Pseudomonas*, notably the phytopathogen *Pseudomonas syringae*, is also able to synthesize the capsular polysaccharide levan through the extracellular enzyme levansucrase (Li and Ullrich, 2001).
Initially identified as an additional determinant for biofilm formation in enterobacteria (Zogaj et al., 2001), cellulose, is a polysaccharide consisting of a linear chain of several hundreds ß-1,4-linked D-glucose monomers commonly found in bacterial extracellular matrix; its production has been described in *Escherichia coli*, in *Salmonella* strains (Jonas et al., 2007), in *Vibrio fischeri* (Bassis and Visick, 2010) and in different *Pseudomonas* environmental isolates (Ude et al., 2006). An acetylated form of cellulose has also been identified in *P. fluorescens* SBW25 and *Pseudomonas syringae* patovar *tomato* DC3000 strains (Spiers et al., 2003; Ude et al., 2006).
The genes responsible for the synthesis of yet another EPS, poly-*N*-acetylglucosamine (PNAG), are present in a large number of Gram-negative bacteria including *E. coli*, *Yersinia pestis*, *Actinobacillus pleuropneumonea*, *Bordetella bronchiseptica* (Whitney and Howell, 2013) and in Gram-positive bacteria such as *Staphylococcus epidermidis* and *S. aureus* (Maira-Litran et al., 2002). PNAG is a homopolymer of ß-1,6-linked *N*-acetyl-D-glucosamine molecules and functions as an important component of the matrix of these bacteria contributing to biofilm formation and persistence during infections (Maira-Litran et al., 2002; Darby et al., 2002; Wang et al., 2004).

2.2 Lipopolysaccharide (LPS)

The lipopolysaccharide (LPS) represents the main component of the outer membrane of Gram-negative bacteria and consists of three different regions: the lipid A, which is hydrophobic and forms part of the lipid bilayer, the core oligosaccharide, and the O-antigen or O-side chain (Figure 3). LPS was initially studied for its ability to act as endotoxins and to stimulate a strong immune response in animals. In addition, LPS is also involved in bacterial adhesion to intestinal cells, represents an important virulence factor in organisms like *Vibrio cholerae*, and is considered a specific determinant in biofilm formation (Chatterjee and Chaudhuri, 2006). Indeed, depending on the strain, the presence of the O-antigen confers a negative charge that generate attractive or repulsive forces with the adhesion surface (Jucker et al., 1996). Studies in *Pseudomonas aeruginosa* indicate that changes in LPS phenotype affect adherence properties and influence biofilm formation (Rocchetta et al., 1999), while knock-out mutations in *rfaG*, *rfaP* and *galU* genes, which are

involved in LPS core biosynthesis, negatively affect adhesion to polystyrene surfaces of the *E. coli* W3100 strain; a similar effect has been also observed in *galU* and *galE* mutants of *Vibrio cholerae* (Nesper et al., 2001), where the O-antigen promotes biofilm formation (Chatterjee and Chaudhuri, 2006). However, in these mutants, the loss of adhesion seems to be caused by the alteration of type I fimbrae and/or flagella associated with these mutations (Genevaux et al., 1999). Furthermore, *galU* and *galE* genes code for enzymes involved in carbohydrate moieties activation (UDP-glucose and UDP-galactose), which are often involved in synthesis of different surface structures of bacteria (Nesper et al., 2001), suggesting that reduced biofilm formation of mutants could be due to the lack of EPS production, rather than to a direct role of LPS in adhesion. In *E. coli* W3100 grown in anoxic conditions, the ability to adhere to hydrophilic surfaces is hindered by LPS overproduction. At the same time, inactivation of the *waaQ* gene, which belongs to LPS core biosynthesis operon, stimulates adhesion both in presence or absence of oxygen, suggesting a negative role of the lipopolysaccharide (Landini and Zehnder, 2002). On the contrary, strains defective in LPS synthesis, such as *Klebsiella pneumoniae*, *Proteus mirabilis*, *Serratia marcescens*, have a reduced capacity to adhere to uroepithelial cells, as well as to form biofilms (Izquierdo et al., 2002).

Although a clear role for LPS in adhesion and biofilm formation has not yet been identified, data suggest that it can contribute in different ways to the adhesion properties of the cell, by either attracting or repulsing the cell toward the surface or by simply creating a stable membrane framework which permits the correct assembly of other adhesion structures.

2.3 Flagella

Flagella are helicoidal rotary appendages driven from a motor at the base, with a filament acting as a propeller. The bacterial flagellum consists in three distinct domains: the basal body, the hook and the filament (Bardy et al., 2003). This structure is primarily involved in cellular motility and chemotaxis, but it also has a sensory function in detecting environmental wetness (Q. Wang et al., 2005).

The flagellum is composed of over 20 proteins species with another 30 proteins required for its regulation and assembly, thus representing one of the most complex prokaryotic organelle (Bardy et al., 2003).

Like LPS, flagella seem to have a dual nature during biofilm formation and their role is unclear.

Biofilm and motility seem to be correlated; indeed, strains with the most robust biofilm formation also display the most vigorous motility as planktonic cells (Wood et al., 2006). A functional

flagellar apparatus appears to be important in the initial stages of biofilm formation stabilizing the contact between the surface and the cell, aiding bacteria to overcome the repulsive forces generated by electrostatic interactions (Genevaux et al., 1996; Pratt and Kolter, 1998). Furthermore, mutations in genes required for flagellar biosynthesis, hinder the capacity to adhere to solid surfaces and to form biofilm in *E. coli* and *P. aeruginosa* (Pratt and Kolter, 1998; O'Toole and Kolter, 1998). A similar effect has been observed in flagellum deficient mutants of *Vibrio cholerae* O139 (Watnick et al., 2001). However, the same authors proved that the absence of the flagellar structure constitutes a signal to increase exopolysaccharide production. Although exopolisaccharide synthesis is required for the correct development of a three-dimensional biofilm, inappropriate exopolysaccharide production leads to inefficient colonization of mouse intestinal epithelium, suggesting that flagellum is not directly involved in biofilm formation (Watnick et al., 2001). Furthermore, in some cases, motility deficient mutants of *Escherichia coli* are still able to form a normal biofilm (Prigent-Combaret et al., 2000).

Recent pieces of evidence suggest that during biofilm formation flagellar genes expression and flagellum activity are turned off through a molecular mechanism that involves the second messenger c-di-GMP. During transition from planktonic cells to biofilm, the second messenger c-di-GMP accumulates to a high level inside the cell favouring the expression of adhesion genes, while repressing the activity of the flagellum binding the "motor-brake" protein YcgR (Fang and Gomelsky, 2010; Paul et al., 2010). Moreover, it has been demonstrated that binding of c-di-GMP to the regulatory subunit of the cellulose synthase complex promotes the biosynthesis of this extracellular polysaccharide that accumulates around the cell impeding the flagellum rotation without affecting flagellum gene expression, exportation, or assembly. Thus, c-di-GMP is able to inhibit motility with a coordinated action on the flagellum motor and on the rotation movement itself (Zorraquino et al., 2013) (Figure 4).

Depending on the bacterial species under study, the regulation of motility during biofilm formation is quite different. However, comparing data coming from *Bacillus*, *Pseudomonas*, *Vibrio*, and *Escherichia,* a common trend emerges: the motility-to-biofilm transition, if necessary, likely involves two steps (Guttenplan and Kearns, 2013): in the short term, flagella are functionally regulated to either inhibit rotation or modulate the basal flagellar reversal frequency; over the long term, flagellar gene transcription is inhibited and in the absence of *de novo* synthesis, flagella are diluted to extinction through growth (Guttenplan and Kearns, 2013).

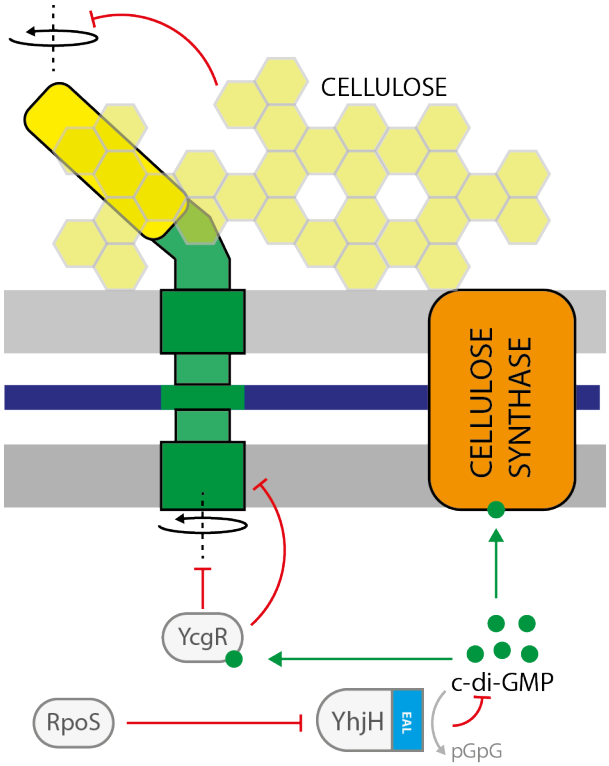

Figure 4. In *E. coli* the second messenger c-di-GMP modulates motility acting on the rotation movement with a coordinated action. YhjH phosphodiesterase degrades c-di-GMP to a linear molecule of pGpG. The expression of *yhjH* gene is higher during exponential phase while its transcription is repressed at the onset of stationary phase by the alternative sigma factor RpoS. A lower activity of YhjH protein promotes the accumulation of c-di-GMP, which is bound by the YcgR "motor-brake" protein that interacts with the flagellar motor inhibiting the rotatory movement. At the same time high concentrations of the second messenger c-di-GMP stimulate cellulose synthesis through the activation of the cellulose synthase complex. EPS accumulation on cellular surface creates a physical barrier that interferes with the flagellum rotation.

2.4 Extracellular DNA (eDNA)

Extracellular DNA (eDNA) is an important biofilm determinant in a number of bacterial species and is found on the biofilm substratum in grid-like patterns. It was suggested that bacteria could initially use the eDNA as a grid that allows their motility using type IV pili and to form a peculiar mushroom-like structure (Karatan and Watnick, 2009). *Pseudomonas aeruginosa* matrix contains significant amounts of eDNA, which are necessary for biofilm integrity and stability (Matsukawa and Greenberg, 2004; Whitchurch et al., 2002). Indeed, exposure of *P. aeruginosa* (both laboratory strains and clinical isolates) to enzymes with DNase activity prevents biofilm formation and dissolves preformed biofilms (Whitchurch et al., 2002; Nemoto et al., 2003). Similar observations were reported for other bacterial biofilms formed by both Gram-positive and Gram-negative bacteria (Nijland et al., 2010; Shields et al., 2013). As observed in *Enterococcus faecalis* biofilms, the source of eDNA seems to be chromosomal DNA, which is released as a result of an active lysis of a portion of cells in the biofilm through the activity of the extracellular protease GelE (Thomas et al., 2008). A similar process has been described in *Staphylococcus epidermidis* and *S. aureus* (Qin et al., 2007; Rice et al., 2007), where lysis is achieved through the activity of AltE autolysin and CidA murein hydrolase, respectively. Therefore, cell lysis and release of genomic DNA may be a

common mechanism that leads to the presence of DNA inside biofilm matrix (Karatan and Watnick, 2009).
The cues that regulate eDNA release are still unknown, but in *P. aeruginosa* regulation of lysis is connected to quorum-sensing signalling, as mutants in quorum-sensing genes *lasI* and *rhlR* release less eDNA (Allesen-Holm et al., 2006).

2.5 Outer membrane proteins (OMPs)

Integral outer membrane proteins (OMPs) span the outer membrane and are generally characterized by an anti-parallel ß-barrel folding (Koebnik et al., 2000). OMPs seem to mediate mainly the adhesion to biotic surfaces, rather than to abiotic ones. Indeed, *P. aeruginosa* OmpF protein works as an adhesin in binding to lung epithelial cells in culture (Azghani et al., 2002), the outer membrane protein OmpX mediates adhesion to mammalian cells in *E. coli* (Vogt and Schulz, 1999), while YOP1 protein of *Yersinia enterocolitica* promotes adherence to human epithelial cells (Heesemann and Grüter, 1987). However, it has been suggested that in response the interaction with abiotic surfaces, OMPs may affect other surface structures involved in adhesion rather than play a direct role in cell-surface contact. Indeed, type 1 fimbrae-mediate surface contact leads to changes in the outer membrane composition, including reductions in the number of many outer membrane proteins (Otto et al., 2001). Furthermore, inactivation of *ompX* enhances fimbriation and increased EPS production (Otto and Hermansson, 2004), thus suggesting that OmpX likely affects the regulation of different surface structures.

2.6 Pili and fimbriae

Many bacterial species, both Gram-positive and Gram-negative, posses long filamentous structures, which extend from their surface and are known as pili or fimbriae. Although in literature the two names are often used to indicate the same structure, pili and fimbrae represent two structurally and functionally different classes of molecules. Fimbriae are always found in higher number on the surface of Gram-negative bacteria and are involved in surface adhesion (Van Houdt and Michiels, 2005), whereas pili are generally found in very limited numbers (usually no more than two) on the surface of both Gram-positive and Gram-negative bacteria and are involved in biofilm formation, phage transduction, DNA uptake and in twitching motility (Proft and Baker, 2009).

In Gram-negative bacteria, pili are typically formed by several hundreds of non-covalent homopolymerized 15-25 kDa subunits, called pilins, which form the pilus shaft (Proft and Baker, 2009). Even though not all pili are contractile, it seems that their capacity to retract could be important in biofilm formation favouring the approach of bacteria. In *E. coli*, conjugative F-pili are used to establish tight cell-cell connections, promoting genetic material transfer between donor and recipient cells. Type F pili are encoded by natural conjugative plasmids, which thus direct the expression of biofilm factors as a part of a coordinated strategy aimed to their propagation (Ghigo, 2001). In *P. aeruginosa*, type IV pili are important for biofilm formation; strains defective for their production are unable to form microcolonies and cannot progress beyond the initial adhesion step (O'Toole and Kolter, 1998). In addition, these same structures are able to bind extracellular DNA present in the biofilm matrix suggesting a role in biofilm maturation processes (van Schaik et al., 2005). In *V. cholerae* the type IV-like toxin-coregulate pilus (TCP) is involved in the host intestine colonization, while attachment to abiotic surfaces of this bacterium seems to be mediated by the mannose-sensitive hemagglutin (MSHA) pilus (Watnick et al., 1999). *Vibrio parahaemolyticus* RIMD2210633 synthetizes two sets of type IV-A pilus, which are involved in biofilm formation; while one set, similar to *Vibrio cholerae* chitin-regulated pilus (ChiRP) mediates only cell-to-cell adhesion, the second set that is homologous to the MSHA pilus is responsible for the sole surface adhesion (Shime-Hattori et al., 2006).

Fimbriae are generally shorter than pili and have been associated with attachment to host tissues in several pathogenic *E. coli* strains (Finlay and Falkow, 1997). The most common adhesins found in *E. coli* isolates as well as in other *Enterobacteriaceae*, such as *Salmonella* strains, are Type 1 fimbriae that are 7-nm wide and approximately 1-μm long (Van Houdt and Michiels, 2005). Type 1 fimbriae are encoded by *fim* operons and are subjected to phase variable expression due to the inversion of a DNA fragment present in the promoter of *fim* genes that depends on the activity of the two recombinases FimB and FimE (Klemm, 1986; Gally et al., 1996). Type 1 fimbrae have been proved to be important during adhesion to abiotic surfaces such as PVC (Pratt and Kolter, 1998), Pyrex (Beloin et al., 2004) and glass wool (Ren et al., 2004). *E. coli* strains often are able to synthetize other types of fimbriae, whose involvement in biofilm formation has been studied to a lesser extent. For instance, enteroaggregative *E.coli* (EAEC) forms a thick biofilm on the intestinal mucosa, which is mediated by aggregative adherence fimbrae AAF/I and AAF/II (Sheikh et al., 2001).

A particular type of fimbriae, curli fibres (also known as thin aggregative fimbriae, or TAFi, in *Salmonella*), plays a pivotal role in cellular adhesion during biofilm formation in enterobacteria and

are one of the main focuses of my research; therefore, their structure, genetics and regulation will be discussed thoroughly in Chapter 4.

2.7 Autotransporter proteins

Autotransporter proteins represent another group of surface exposed proteins involved in bacterial adhesion. In Gram-negative bacteria, autotransporter proteins are secreted via type Va secretion systems (Henderson et al., 2004) and are characterized by a primary sequence that contains all the information needed to traverse the outer membrane. A recent study has identified over 500 Type Va autotransporter proteins sequences on the NCBI database, suggesting that this kind of structures are well distributed in bacterial genomes (Junker et al., 2006). All the autotransporter proteins are structurally characterized by the presence of three different domains: an N-terminal signal which mediates the transport across the cytoplasmic membrane, a surface-localized domain, termed passenger domain, and a C-terminal domain, termed the translocation domain that facilitate secretion through the outer membrane (Wells et al., 2007). While the translocation domain is conserved, the surface-localized passenger domain displays a high degree of variability between different autotransporter proteins. Thus, depending on the passenger domain type, autotransporter proteins are involved in adhesion, autoaggregation, biofilm formation, or cytotoxicity (Henderson and Nataro, 2001). For instance, Peractin of *B. pertussis* functions as a major virulence factor mediating the binding to the lung epithelium (Everest et al., 1996), while the adhesion and penetration protein Hap from *Haemophilus influenza*e is associated with attachment and entry into epithelial cell, attachment to extracellular matrix proteins, and bacterial aggregation and microcolonies formation (St Geme et al., 1994; Fink et al., 2002, 2003).

Antigen 43 (Ag43), the product of the *flu* gene, is one of the most studied autotransporter proteins and one of the most represented proteins on the surface of *E. coli* cells. This autotransporter protein promotes cell autoaggregation through Ag43-Ag43 interactions and stimulates bacterial biofilm formation at the microcolony stage (Danese et al., 2000; Kjaergaard et al., 2000). When assembled, Ag43 forms 10 nm-long structures, hence the presence of other extracellular structures thicker or longer than 1 μm, such as capsular polysaccharides or Type 1 fimbriae, inhibit the Ag43-mediated autoaggregation blocking the physical interaction between two Ag43 shafts (Schembri et al., 2004; Hasman et al., 1999). Similar to Type 1 fimbrae, Antigen 43 undergoes phase-variable expression modulated by the opposite activities of the Dam methylase enzyme and the transcriptional regulator OxyR (Haagmans and van der Woude, 2000). In addition, in some uropathogenic *E. coli* strain,

Ag43 has been found to be glycosylated with heptosyl residues but this modification is not required for adhesion (Sherlock et al., 2006)

E. coli is able to produce at least two other autotransporter proteins: the adhesin involved in diffuse adherence (AIDA) and the TibA protein. AIDA, a glycosylated protein, is associated with intestinal cells adherence of diarrhea-causing *E. coli* strains (Benz and Schmidt, 1989) and, like Ag43, is a self-recognizing protein that enhances biofilm formation and autoaggregation (Sherlock et al., 2004). TibA protein, encoded by *tibA* gene, shows homology to both AIDA and Ag43, and in contrast to Antigen 43 requires glycosylation for epithelial cell adherence and invasion (Lindenthal and Elsinghorst, 2001).

Table 1. Extracellular structures involved in cellular adhesion and biofilm formation. The table summarizes the examples described in Chapter 2.

Extracellular structure	Characteristics and Functions	Examples	Found in	References
Extracellular polysaccharides (EPS)	Involved in cell envelope integrity maintenance, cellular desiccation prevention, promotion of the correct shaping and maturation of biofilm, protection from environmental stresses and bactericidals.	Alginate	*Pseudomonas* spp.	(Deretic et al., 1990)
		PEL	*Pseudomonas* spp.	(Matsukawa and Greenberg, 2004)
		PSL	*Pseudomonas* spp.	(Jackson et al., 2004)
		Levan	*P. syringae*	(Li and Ullrich, 2001)
		Cellulose/Acetylated cellulose	*Pseudomonas* spp.	(Ude et al., 2006)
			E. coli, *Salmonella* spp.	(Jonas et al., 2007)
			V. fischeri	(Bassis and Visick, 2010)
		PNAG	*E. coli*	(Whitney and Howell, 2013)
			Y. pestis	
			A. pleuropneumonea	
			B. bronchiseptica	
			S. epidermidis, S. aureus	(Maira-Litran et al., 2002)
Lipopolysaccharide (LPS)	Main component of the outer membrane of Gram-negative bacteria. No clear role in adhesion and biofilm formation identified. Facilitates adhesion by either attracting or repulsing the cell toward the surface or by creating a stable membrane framework.		Gram-negative bacteria	(Chatterjee and Chaudhuri, 2006) (Jucker et al., 1996) (Rocchetta et al., 1999) (Landini and Zehnder, 2002) (Izquierdo et al., 2002)
Flagellum	Primarily involved in cellular motility and chemotaxis, No clear role during biofilm formation. Flagellum is important during the initial phases of biofilm formation stabilizing the contact between the surface and the cell. During biofilm maturation its production is turned off.		Gram-positive and Gram-negative bacteria	(Genevaux et al., 1996) (Pratt and Kolter, 1998) (Guttenplan and Kearns, 2013)
Extracellular DNA (eDNA)	Important for structural stability of bacterial communities and for type IV pili-mediated motility inside biofilms.		*P. aeruginosa*	(Whitchurch et al., 2002)
			E. fecalis	(Thomas et al., 2008)
			S. epidermidis	(Qin et al., 2007)
			S. aureus	(Rice et al., 2007)

Extracellular structure	Characteristics and Functions	Examples	Found in	References
Outer membrane proteins (OMPs)	Mediate adhesion to biotic surfaces such as human epithelium.	OmpF	*P. aeruginosa*	(Azghani et al., 2002)
		OmpX	*E. coli*	(Vogt and Schulz, 1999)
		YOP1	*Y. enterocolitica*	(Heesemann and Grüter, 1987)
Pili	Found in very limited numbers (usually no more than two) on the surface of both Gram-positive and Gram-negative bacteria. Involved in biofilm formation, phage transduction, DNA uptake and in twitching motility.	Type F pili	*E. coli*	(Ghigo, 2001)
		Type IV pili	*P. aeruginosa*	(van Schaik et al., 2005)
		TCP pilus MSHA pilus	*V. cholerae*	(Watnick et al., 1999)
Fimbriae	Found in high number on cell surface, are involved in surface adhesion and cell-cell interaction.	Type 1 fimbrae	*E. coli*, *Salmonella* spp.	(Van Houdt and Michiels, 2005)
		AAF/I and AAF/II	enteroaggregative *E.coli* strains	(Sheikh et al., 2001)
			E. coli. *Salmonella* spp.	(Olsén et al., 1989)
			M. tuberculosis	(Alteri et al., 2007)
		Curli fibres and similar β-amyloid fibres	*B. subtilis*	(Romero et al., 2010)
			environmental isolates belonging to	(Larsen et al., 2007)
Autotransporter proteins	Self-assembling extracellular structures involved in bacterial adhesion, autoaggregation, biofilm formation, and cytotoxicity.	Peractin	*B. pertussis*	(Everest et al., 1996)
		Hap	*H. influenzae*	(St Geme et al., 1994; Fink et al., 2003)
		Flu (Ag43)	*E. coli*	(Danese et al., 2000)

3 Regulation of biofilm formation

Biofilm formation involves a considerable amount of physiological and structural changes in the bacterial cell and consequently requires a significant consumption of energy. Furthermore, biofilm is frequently formed during adverse growth conditions, when nutrient availability is scarce, thus it is pivotal for the cell to correctly integrate environmental and physiological stimuli in order to determine whether keep progressing toward colony formation or stop in early phases. This decisional process requires both specific and global regulators that form a complex and integrated network. Interestingly, however, few biofilm-dedicated regulatory pathways have been discovered. Indeed, as already discussed in a previous paragraphs, it was proposed that modifications in biofilm structure and amount depend on central metabolic changes due to the modified environment and not a result of a environmental signal that follow a specific pathway (Monds and O'Toole, 2009). In the next sections I will review the common mechanisms that regulate the biofilm formation process, focusing my attention especially on metabolic signals, as they are central to my experimental work (see Table 3, pag. 38-40 for a summary).

3.1 Environmental signals

Several different environmental signals that influence biofilm formation both directly and indirectly have been characterized, though many of them still poorly understood.

Nutritional status of the environment is one of the most important cues for biofilm formation, perhaps because both the formation and the dispersion of a biofilm require high energy consumption. However, different bacterial species may respond in the opposite way to the same nutritional state. Indeed, *Salmonella enterica* and the closely related *E. coli* form biofilm in response to nutrient limitation (Gerstel and Römling, 2003), while *V. cholerae* biofilm formation is stimulated in nutrient-rich conditions (Yildiz et al., 2004). In particular, it seems that glucose availability is a key determinant in the decision to switch to biofilm, promoting its formation in some organisms, such as *S. aureus*, *S. epidermidis* and *V. cholerae* (Dobinsky et al., 2003; Lim et al., 2004), enteroaggregative *E. coli* strains (Sheikh et al., 2001) and *S. enterica* (Bonafonte et al., 2000), while playing a negative role in other, *i.e. E. coli* K-12 (Debra W Jackson et al., 2002). Glucose-mediated regulation is often achieved through two distinct levels of regulation: at transcriptional level through the cAMP/CRP and Cra regulons that act independently in order to

modulate the carbon flow through the different metabolic pathways of energy metabolism, and at post-transcriptional level through the activity of the Carbon storage regulator A (CsrA) (see Paragraph 3.4.2).

Oxygen is another cue that influences cellular adhesion and biofilm formation. In oxygen-limiting condition *P. aeruginosa* forms more biofilm, and shows increased antibiotic tolerance and alginate biosynthesis (Schobert and Tielen, 2010). On the contrary, a microaerophilic environment negatively affects *E. coli* adherence capacity on hydrophilic substrates (Landini and Zehnder, 2002). Moreover, in the model organism *E. coli* K-12 str. MG1655 two important adhesion determinants, namely curli fibres and PNAG, are regulated by the oxygen sensory system DosP/DosC, which probably adjust levels of the second messenger c-di-GMP in response to oxygen availability (Tagliabue, Maciag, et al., 2010; Tagliabue, Antoniani, et al., 2010).

Iron is another essential and yet scarce nutrient for bacteria. Most of the iron in the environment either resides stably in inorganic complexes or tightly bound to molecules called siderophores (Miethke and Marahiel, 2007). As seen for other environmental signals, the effects of iron limitation on biofilm formation can vary considerably in different bacteria. For example, in *P. aeruginosa*, high concentrations of iron induce expression of adhesion factors, while scarce levels of the metal inhibit biofilm formation process (Bollinger et al., 2001). Similarly, *V. cholerae* biofilm formation is reduced upon iron-limiting conditions (Mey et al., 2005).

Like iron, inorganic phosphate has important involvement in biofilm formation and phosphate-limiting condition inhibits its formation in *P. fluorescens* (Monds et al., 2007). This signal is transduced by the PhoPQ two-component system (TCS) that regulates conditional expression of RapA, a c-di-GMP phosphodiesterase, resulting in lower intracellular level of the second messenger c-di-GMP (see Chapter 3.4.4) that prevent the secretion of the adhesion LapA (Monds et al., 2007). The Pho TCS also mediates response to phosphate in *Agrobacterium tumefacens*, but in the opposite way: phosphate limitation enhances biofilm formation of this plant pathogen (Danhorn et al., 2004).

Two-component systems (TCS), also known as Two-component regulatory systems (TCRS), represent one of the main regulatory mechanisms for the response to environmental signal found in bacteria. TCS are typically constituted by a sensor protein, usually found in the cytoplasmic membrane, sensing the environmental cue, and a response regulator able to bind specific DNA sequences, thus triggering a coordinated response to the signal (Mikkelsen et al., 2011). Several TCS are involved in biofilm regulation in different bacteria: for instance, in *E. coli*, osmolarity affects cell-surface interactions promoting or inhibiting the process of biofilm formation, and its regulation is achieved through the activity of EnvZ/OmpR two-components system that mediate the

global response to osmolarity (Cai and Inouye, 2002). Interestingly, in this bacterium, high concentrations of NaCl either abolish curli production (Jubelin et al., 2005) or stimulate the synthesis of PNAG (Goller et al., 2006), another adhesion factor, thus indicating that different matrix components might be preferred in different environmental conditions.

Temperature is another key signal, especially for pathogens or commensal bacteria, in which temperature changes indicate the transition between the external environments, where the temperature is usually lower, and the host, where temperature is higher and more stable. Thus, depending on the bacterium, temperature has different effects on biofilm formation. Indeed, in the non-pathogenic bacterium *E. coli*, temperature higher than 32°C, typical of the host, inhibit the synthesis of curli adhesins (Olsén et al., 1993), while at the same temperature, curli are stimulated in pathogenic enteroinvasive (EIEC) or enteroemorragic (EHEC) strains of *E. coli* (Cookson et al., 2002).

Several symbiotic organisms, both commensal and pathogenic, modulate biofilm production in response to external host-derived signals. For example, the presence in the small intestine of bile, a potent detergent that kills bacteria by solubilizing their membrane, stimulates biofilm formation in the pathogen *V. cholerae* (Hung et al., 2006) and in the beneficial *Lactobacillus* strains (Ambalam et al., 2012). Another example is the response of *P. aeruginosa* to hydrogen peroxide (H_2O_2), a chemical produced by the host immune system, which triggers the production of alginate and the consequent biofilm formation in the opportunistic pathogen (Mathee et al., 1999)

3.2 Intracellular signals and metabolic cues

Products of primary or secondary metabolism may function as intracellular signals molecules that influence extracellular structures formation. Indole, a product of the enzyme tryptophanase, accumulates inside the cell and seems to have a stimulatory effect on biofilm formation. Indeed, inhibition of tryptophanase prevents biofilm formation in a number of clinical isolates of *E. coli*, *Klebsiella oxytoca, Providencia stuartii*, *Citrobacter koseri*, *Morganella morganii*, and *Haemophilus influenzae* (Martino et al., 2003). Furthermore, a mutation that prevented *V. cholerae* biofilm formation was reversed by the addition of exogenous indole (Mueller et al., 2007), suggesting that indole can act as a signal molecule (Wang et al., 2001).

Recently, has been observed D-amino acids accumulate in the supernatant of stationary phase cultures and are important in regulating peptidoglycan composition, amount, and strength, both via their incorporation into the polymer and by regulation of enzymes that synthetize and modify it

(Lam et al., 2009). In *Bacillus subtilis*, incorporation of D-amino acids in the cell wall promotes the release from the peptidoglycan of the protein TasA, which is required for the structural maintenance of the bacterial community (Romero et al., 2010), thus leading to biofilm disassembly (Kolodkin-Gal et al., 2010). This release is mediated by an adaptor protein, TapA, which forms D-amino acid-sensitive foci in the cell wall (Romero et al., 2011). Furthermore, it has been proved that a D-amino acid mix is able to inhibit biofilm formation of both Gram-positive and Gram-negative bacteria, suggesting that D-amino acids may be a common strategy to regulate biofilm dispersion (Kolodkin-Gal et al., 2010).

Polyamines, such as putrescine and spermidine, are organic compounds essential for growth that contain two or more amine groups and are positively charged at neutral pH. Several pieces of evidence suggest that polyamines may function as extracellular and/or metabolic signals that modulate biofilm formation. For example, exogenous norspermidine increases *V. cholerae* biofilm formation by a mechanism that requires two proteins, NspS and MbaA (Karatan et al., 2005). In response to norspermidine NspS, a periplasmic sensory protein, interacts and modulates the activity of MbaA, a protein involved in c-di-GMP second messenger metabolism and in biofilm repression (Bomchil et al., 2003). It was proposed that norspermidine-NspS complex decreases the repression of biofilm formation inducing a conformational change in the MbaA protein that reduces its activity (Karatan et al., 2005). The same system is also responsible for responding to exogenous and endogenous spermidine, which, on the contrary, inhibits biofilm formation (McGinnis et al., 2009). PotD, an NspS homolog in *E. coli*, has been recently described as a modulator of biofilm formation, suggesting that polyamines could regulate biofilm formation in this bacterium through a similar mechanism (Zhang et al., 2013).

In *Y. pestis* mutants unable to synthetize endogenous putrescine are impaired in biofilm formation. This defect can be rescued in a dose-dependent manner by supplementation of the growth medium with putrescine, suggesting that biofilm formation can be triggered by both endogenous and exogenous putrescine (Patel et al., 2006). Indeed, absence of putrescine leads to a reduction in the levels of HmsR, HmsS, and HmsT proteins, which are necessary for biofilm formation in this pathogen (Wortham et al., 2010). This effect is achieved through the modulation of the translation of *hmsT* and *hmsR* mRNAs. Interestingly polyamines are often found in complexes with RNA suggesting that they also have the ability to influence protein expression through translation. Indeed, in *E. coli*, polyamines addition induces a conformational change in the *oppA* RNA that aids the formation of an initiation complex and increases the rate of translation (Yoshida et al., 1999). Therefore a similar regulation has been proposed for *hms* proteins (Wortham et al., 2010).

Polyamines also modulate biofilm formation by directly targeting components of the extracellular matrix. In *B. subtilis*, norspermidine and norspermine seem to directly interact with the negatively-charged extracellular polysaccharides network promoting its collapse and the release of polymers, thus leading to biofilm dispersal (Kolodkin-Gal et al., 2012). Therefore, although polyamines regulate biofilm formation in a broad range of bacteria, the mechanism of action could be different as these molecules can directly act on transcription, translation and on extracellular structures stability.

3.3 Quorum-sensing

Quorum-sensing (QS) represents a form of cell-cell communication based on small diffusible molecules called autoinducers, which allow bacteria to coordinate gene expression in a cell density-dependent manner. Quorum-sensing mechanisms play a crucial role in biofilm formation and its maintenance; particularly, they mediate the transition from microcolony to mature biofilm (Hammer and Bassler, 2003; Vuong et al., 2003; Ueda and Wood, 2009). Indeed, studies carried out in *P. aeruginosa* show that, although biofilm is not completely impaired, mutants lacking the autoinducer form a thinner and less structured biofilm, which is more susceptible to antibiotic (Davies et al., 1998).

In addition to biofilm formation, quorum-sensing is also involved in other processes, such as virulence factors production of both Gram-negative and Gram-positive bacteria (Rutherford and Bassler, 2012), regulation of antibiotic production in *Streptomyces* spp., conjugation in *Enterococcus fecalis*, and fruiting body development in *Myxococcus xanthus* (Miller and Bassler, 2001).

The signal molecules are synthesized and secreted during bacterial growth, and accumulate as the population increases until reaching a threshold concentration, defined as “quorum”, which triggers the expression of target genes. Although quorum-sensing is broadly conserved, the molecular mechanism, as well as the chemical nature of the autoinducers, differ significantly between Gram-positive and Gram-negative bacteria (Miller and Bassler, 2001).

In Gram-negative bacteria, all the quorum-sensing systems characterized so far, with the sole exceptions of *V. harveyi* and *M. xanthus*, resemble the canonical quorum sensing circuit of the symbiotic bacterium *V. fischeri*. The system relies on two proteins: an autoinducer synthase and a receptor, usually belonging to the LuxI and LuxR protein families, respectively. LuxI-like proteins are responsible for the biosynthesis of a specific N-Acyl homoserine lactones signalling molecule

(AHL), while LuxR-like proteins bind the cognate autoinducer once it reaches a critical threshold concentration, and activate the transcription of target genes (Fuqua et al., 1994, 1996). In addition, some bacteria are able to synthesize species-specific autoinducers other than AHL, such as quinolones in *P. aeruginosa* (PQS, for *P. aeruginosa* Quinolone Signal; McKnight et al., 2000), and the so called diffusible signal factor (DSF), a fatty acid used as signal molecule by the plant pathogen *Xanthomonas campestris* (Barber et al., 1997).

In contrast, in Gram-positive quorum sensing circuits the signal molecules is commonly constituted by short peptides (5 – 50 amino acids) synthesized directly by ribosomes and often subjected to extensive post-translational modifications (Miller and Bassler, 2001). Binding of signal peptide to the sensor protein in the membrane triggers a signal transduction cascade, resulting in phosphorylation of the cognate response regulator that activates target genes. One of the most studied QS system in Gram-positive bacteria is the *Staphylococcus aureus* accessory gene regulation system (*agr* system), in which the autoinducer-dependent phosphorylation of the AgrA regulator activates a number of genes that are mainly involved in bacterial virulence and in repressing biofilm formation (Vuong et al., 2000; Boles et al., 2004).

In addition to AHL-type and peptide-based autoinducers, both Gram-negative and Gram-positive are able to synthesize a second signal molecule called autoinducers-2 (AI-2), which enables interspecies communication (Pereira et al., 2013). AI-2 is a furanosyl borate ester, one of the few known biomolecules incorporating boron, and is synthesized from proteins belonging to the LuxS family. However, several pieces of evidence suggest that a LuxS-independent pathway exists; indeed, AI-2 activity was reported from *luxS*-deficient extracts supplied with adenosine (Li et al., 2006) and from *E. coli luxS* mutants (Tavender et al., 2008).

Two classes of AI-2 receptors have been characterized to date: proteins belonging to the LuxP family and proteins of LsrB family (Chen et al., 2002; Miller et al., 2004). More recently, a third class of molecules, protein of the ribosome binding protein (RbsB) family showed AI-2 binding capabilities, suggesting that RbsB-family may also act as AI-2 receptors (Armbruster et al., 2011). However, strains lacking LuxP-, LsrB- and RbsB-like receptor are still able to respond to AI-2, suggesting that AI-2 can be bound by additional, not yet identified, receptors (Pereira et al., 2013).

As other QS systems, AI-2 affects different aspects of bacterial physiology. In *Vibrio* spp. it regulates bioluminescence, metalloprotease and siderophores production, type III secretion, virulence factors assembly, matrix synthesis and biofilm formation. In contrast, AI-2 seems to affect virulence, adherence, biofilm formation, swimming and swarming in *E. coli* and *Salmonella* strains. In these bacteria, AI-2 has been shown to work also as a chemoattractant regulating

bacterial motility (Pereira et al., 2013). In *E. coli*, flagellar regulation by AI-2 seems to be mediated by the activity of the protein MqsR (González Barrios et al., 2006).

3.4 Global regulators

Responding to the changing environment can require extensive resetting of gene expression. This is achieved through the so-called global regulators that allow bacteria to rapidly modulate the expression of a large variety of unrelated genes or operons scattered over the genome. Thus, this definition includes a broad spectrum of effectors including protein, non-coding RNAs and signalling molecules that can act at transcriptional, post-transcriptional and post-translational level. Clearly, global regulators play a pivotal role in regulating the expression of extracellular structures and the switch between planktonic and sessile life-styles.

3.4.1 Transcriptional regulators

Several different global transcriptional regulators have been identified so far. In *E. coli,* nucleoid associated proteins, such as H-NS, Fis, IHF, and HU (Arnqvist et al., 1994; Sheikh et al., 2001; Gerstel and Römling, 2003; Hansen et al., 2008), and transcriptional factors, such as RpoS, Lrp, RfaH (Landini et al., 2013; Beloin et al., 2006; Hung et al., 2002; van der Woude et al., 1992) control the production of biofilm determinants and regulate the progression through the different phases of biofilm formation. Among the listed proteins H-NS and RpoS are the two most studied and well characterized.

The head-stable nucleoid-structuring (H-NS) protein is one of the twelve nucleoid-associated proteins described to date in *E. coli*. The relative levels of the different nucleoid-associated proteins in any given cell is not fixed. This is because the genes that encode these proteins are subject to individual and complex regulation. Any set of growth conditions will produce a characteristic profile of nucleoid-associated proteins (Dorman, 2004). The H-NS protein is expressed at a relatively constant level through the growth, possibly being subjected to a small increase in early stationary phase (Dorman, 2004). H-NS binds to curved DNA, which is commonly found at promoters, exercising a negative effect on transcription (Dorman, 2004). When bound to DNA, H-NS prevents association of σ^{70}-RNAP complex to promoters, hence, inhibiting transcription of target genes during exponential growth. However, RNA polymerase associated with the alternative sigma factor σ^{S}, mainly expressed during stationary phase, is able to overcome H-NS repression

resulting in the expression of stationary phase-specific genes. This phenomenon, known as "exponential silencing", controls, among others, the expression of curli fibres, the main adhesion determinants in many *E. coli* strains, limiting their synthesis to the a specific growth phase (Arnqvist et al., 1994). In addition to curli, in *E. coli* K-12, H-NS is responsible of the repression of approximately 5% of genes including genes involved in flagellum (Soutourina et al., 1999) and LPS biosynthesis (Landini and Zehnder, 2002), preventing initial adhesion to hydrophilic surfaces and subsequent biofilm formation. Furthermore, H-NS negative role on biofilm formation has been also observed in other Gram-negative bacteria, such as *Y. enterocolitica* (Bläbel et al., 2013) and *Vibrio cholerae*, where the global regulator represses Vibrio exopolysaccharide (*vps*) biosynthesis genes (Wang et al., 2012).

Sigma factors represent one of the most common classes of global regulators. They direct RNA polymerase binding to specific DNA sequences in gene promoter regions, thus allowing correct transcription initiation. In addition to the major housekeeping σ^{70} factor, *E. coli* synthesizes six other sigma factors that regulate the most diverse bacterial responses to environmental cues. RpoS (σ^{S}) is the key regulator of general stress response and, in *E. coli*, it has been proposed that its regulon comprises ca. 500 genes, thus accounting for about 10% of the complete genome (reviewed in Landini et al., 2013). RpoS mediates the response to osmotic shock, oxidative stress, acid stress, regulating cellular metabolism, protein processing, transcriptional regulators, transporters and membrane proteins among others (Landini et al., 2013). In addition, it also directly controls genes involved in biofilm formation such as curli fibres and cellulose (Gerstel and Römling, 2003), and genes connected to the biosynthesis of the signal molecule c-di-GMP, which mediates the transition from planktonic to sessile communities (Sommerfeldt et al., 2009). Indeed, *E. coli* mutants in *rpoS* either form thinner biofilm when grown in continuous cultures (Adams and McLean, 1999) or are not able to establish biofilms (Schembri et al., 2003). RpoS seems to be connected to biofilm formation also in *P. aeruginosa* where its expression appears to be related to quorum-sensing system through mutual control (Whiteley et al., 2000). However, the same authors reported that in *P. aeruginosa* biofilm, expression of RpoS is repressed and that *rpoS*-deficient mutants form biofilm better than wild type cells (Whiteley et al., 2000). Consistent with this findings, RpoS negatively regulate expression of type I fimbrae in *E. coli*, which can also mediate biofilm formation (Dove et al., 1997). Thus, RpoS can affect biofilm formation both positively and negatively; the final output could be the result of the integration of the complex signalling network that is known to control the expression of this global regulator (Landini et al., 2013).

3.4.2 Post-transcriptional regulators

Small noncoding RNAs (sRNAs) are major players in post-transcriptional global regulation. The vast majority of sRNAs acts by base pairing with their mRNA targets with the help of the Hfq RNA chaperone, thereby altering the translation or stability of target mRNAs. Interestingly, a single sRNA can interact with multiple targets resulting in a drastic modification of the cell physiology. For example, in a recent work, bioinformatics target prediction revealed a total of 309 mRNAs as putative targets of the MtvR sRNAs in the highly epidemic clinical isolates *Burkholderia cenocepacia* J2315. Bacterial strains with MtvR silenced or overexpressed exhibited pleiotropic phenotypes, typical of global regulators, related to growth and survival after several stresses, swimming and swarming motility, biofilm formation, resistance to antibiotics, and ability to colonize and kill the host (Ramos et al., 2013). Furthermore, a number of sRNAs is also able to modulate the production of other global regulators; for example, the DsrA sRNA regulates RpoS and H-NS expression, promoting the production of the former and inhibiting the synthesis of the latter (Lease and Belfort, 2000), and therefore affecting biofilm development. This layered network of global regulator allows bacteria to express a vast portion of its genome in response to environmental signals without losing the fine-tuning capabilities usually achieved through the action of specific regulators.

A second group of sRNAs binds to proteins. The best-studied example in *E. coli* is CsrB, a sRNAs that interact and modulate the activity of the post-transcriptional global regulator CsrA. CsrA is a small protein of 61 amino acids that binds to mRNAs and regulates their stability and translation mostly acting as a repressor. As a global regulator, CsrA regulates multiple unrelated pathways, such as central carbon metabolism, oxidative stress response, virulence and pathogenesis (Timmermans and Van Melderen, 2010). In addition, CsrA is a positive regulator of flagella biosynthesis (Wei et al., 2001), while it represses c-di-GMP synthesis and PNAG production, exercising a negative role in the process of biofilm formation (Jonas et al., 2008; X. Wang et al., 2005). CsrA activity is regulated by the binding of two small noncoding RNAs: CsrB and CsrC; both sRNAs contain multiple CsrA binding sites that bind and sequester CsrA, counteracting its activity (Liu et al., 1997; Dubey et al., 2005).

With their ability to affect stability and translation of multiple mRNAs, cold-shock proteins represent another class of possible global regulators that act post-transcriptionally. After temperature downshift, proteins such as CspA, CspB, CspG and PNPase are strongly induced. CspA and its paralogues destabilize mRNA secondary structures, a process that may be crucial for

efficient mRNA translation at low temperatures (Jiang et al., 1997). Even though specific targets of Csp proteins have not been completely identified, recent data suggest that in *Listeria monocytogenes* they are involved in toxin production (Schärer et al., 2013), while in *E. coli* cold-shock proteins work as positive regulators of biofilm formation (Domka et al., 2007).

Polynucleotide phosphorylase (PNPase) is an exonuclease that plays an important role in RNA processing and turnover, being implicated in RNA degradation and in polymerization of heteropolymeric tails at the 3'-end of mRNA (Mohanty and Kushner, 2000, 2006). Inactivation of the *pnp* genes has pleiotropic effects, which include reduced proficiency in homologous recombination and repair, and inability to grow at low temperature. PNPase is also involved in biofilm formation. Indeed, a recent work suggested that it is necessary to maintain bacterial cells in the planktonic mode repressing the *pgaABCD* operon and therefore PNAG production (Carzaniga et al., 2012).

3.4.3 Post-translational regulators

Protein amount and stability is the last level of gene expression regulation. ATP-dependent proteases and chaperones act together to regulate protein stability and folding, often affecting the production of other global regulators such as σ factors and therefore modulating at the same time different aspects of the bacterial life. As an example, *E. coli* Lon protease regulates acid shock tolerance acting on the master regulator GadE (Heuveling et al., 2008) and flagella expression directly degrading the σ^{28} factor (Barembruch and Hengge, 2007). Moreover, Lon degrades RcsA, a positive regulator of capsular polysaccharide production, thus affecting production of colanic acid (Gottesman and Stout, 1991), an extracellular structure important for the determination of biofilm thickness (Prigent-Combaret et al., 2000), in an indirect manner. As already described, RpoS is a global transcriptional regulator that plays an essential role in the general stress response and during biofilm formation. The protein stability is regulated by the ClpXP protease and is mediated the RssB response regulator protein, which is responsible of binding and targeting RpoS to the protease complex (Schweder et al., 1996; Becker et al., 1999). Several different factors regulate RssB binding to the alternative sigma factor. For example, the interaction of the two proteins is modulated upon intracellular concentration of acetyl phosphate, which is directly linked to ATP levels and therefore energy availability (Bouché et al., 1998), and by the anti-adaptor proteins IraP, IraM, and IraD that prevent RssB binding in response to shortages in either phosphate or magnesium, and DNA damage (Bougdour and Gottesman, 2007; Bougdour et al., 2008). ClpXP-

dependent degradation represents the decisive process leading to accumulation of RpoS at the onset of stationary phase of growth and therefore has a deep impact on RpoS-dependent genes and biofilm.

In conclusion, although post-translational global regulators affect biofilm formation processes, the influence seems to be mainly indirect. Indeed, no proteases or chaperones identified to date have as a direct target extracellular structure involved in bacterial adhesion.

3.4.4 Signalling molecules

Modified nucleotides, both linear and cyclic, constitute a particular class of global regulator. Their intracellular levels are regulated by synthases and phosphodiesterases in response to several environmental cues and they act as signalling molecules regulating different bacterial processes such as virulence and biofilm formation among others (Table 2). cAMP, (p)ppGpp, c-di-GMP are the three most studied signalling molecules in bacteria and their connection with biofilm formation will be discussed in the next few paragraphs.

Signal molecule	Phenotype or process regulated
cAMP	Biofilm (-), Virulence (+), Use of secondary carbon sources (+)
(p)ppGpp	Biofilm (-), Transcription (+/-), Translation (+/-), DNA replication (-)
c-di-GMP	Biofilm (+), Virulence (-), Motility (-), Transcription (+/-), Translation (+/-), Proteolysis (-)

Table 2. Phenotypes or processes that are controlled by cAMP, ppGpp, or c-di-GMP signalling molecules. +/- symbols represents positive and negative regulation respectively.

cAMP. Cyclic adenosine monophosphate (cAMP) is a universally used second messenger found in both eukaryotic and prokaryotic cells. cAMP intracellular levels are controlled by the action of adenylyl-cyclases that catalyse its synthesis, and specific cAMP phosphodiesterases, which degrade cAMP to AMP. The cell can sense cAMP levels through cytoplasmatic receptors, of which cAMP-receptor protein (CRP), also known as catabolite gene activator protein (CAP), is the best studied. CRP is activated by the allosteric binding of cAMP, which triggers conformational changes in the tertiary structure of the protein and in its affinity to specific DNA sites (Busby and Ebright, 1999). In *E. coli*, CRP directly controls the transcription of more than 180 genes and mediate the so called catabolite repression process (Busby and Ebright, 1999). In the absence of glucose, intracellular levels of cAMP are high, and cAMP-CRP complex activates genes required for the catabolism of

secondary sugars (lactose, maltose, arabinose). In contrast when glucose is available in the culture media, intracellular cAMP concentration decreases and transcription of catabolic genes is switched off (Görke and Stülke, 2008). Furthermore, cAMP also plays a central role in biofilm-formation, virulence, and type III-secretion systems in many bacteria (Diaz et al., 2011). In *V. cholerae*, during glucose limitation, cAMP-CRP regulates biofilm both directly by down-regulating CdgA, a protein connected with c-di-GMP biosynthesis, and indirectly by affecting the quorum-sensing and virulence regulation of the pathogen (Fong and Yildiz, 2008; Liang et al., 2007). Similarly, cAMP-Vfr, a CRP homologue, was found to regulate the expression of type-III secretion system in *P. aeruginosa* (Wolfgang et al., 2003) and genes belonging to the *las* quorum-sensing system (Fuchs et al., 2010). CRP is also involved in biofilm formation in *E. coli,* regulating the expression of its main adhesion factor (Zheng et al., 2004).

(p)ppGpp. Guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp), collectively known as (p)ppGpp, are synthetized in response to amino acids, phosphates, fatty acids, carbon, and iron starvation. Stress response coordinated by (p)ppGpp leads to a reduction in the growth rate of the cell and is known as "stringent response" (Kalia et al., 2013). Two homologous proteins, RelA and SpoT, control intracellular levels of ppGpp. Starting from GTP and ATP, RelA generates pppGpp, which is then converted to ppGpp. In contrast, SpoT or one of its homologues, can directly synthetize both ppGpp and pppGpp. Furthermore, SpoT is also responsible for the hydrolysis of the two molecules to GDP and PP_i or GTP and PP_i respectively (Kalia et al., 2013). In response to nutrient starvation, (p)ppGpp regulates both directly and indirectly several different processes in bacteria including transcription, translation, DNA replication, virulence and biofilm formation. The signal molecule affects transcription either directly interacting with RNAP or indirectly by interacting with sigma factors and controlling the activity of transcription factors. (p)ppGpp effects on RNAP are enhanced by the interaction of DskA protein with the transcriptional machinery (Paul et al., 2004, 2005): once bound to RNAP, (p)ppGpp inhibits the binding of RNAP to σ^{70}, the housekeeping σ factor, preventing transcription initiation from strong promoters, such as the ones controlling ribosome biogenesis. Through inhibition of core RNAP-σ^{70} assembly, ppGpp increases binding of the core polymerase to other σ-factors, for example σ^S. Thus, stringent response shifts transcription to σ^S-regulon, which, as already described in Chapter 3.4.1, comprises several genes involved in biofilm formation. Moreover, ppGpp promotes σ^S protein stability by inducing expression of the anti-adaptor proteins IraP and IraD that prevent RssB binding to RpoS and the consequent ClpXP-degradation (Bougdour and Gottesman, 2007; Merrikh et al., 2009), further enhancing the effects on σ^S-dependent genes. Besides affecting alternative sigma factors activity, guanosine tetraphosphate influences other global regulators already described in the

previous paragraphs as important for the development of sessile communities. In *E. coli*, (p)ppGpp induces the expression of non-coding regulatory RNAs to regulate the stability of certain mRNAs indirectly via the carbon storage regulator (CsrA) regulatory pathway. Indeed, both ppGpp and the cofactor protein DskA activate transcription of CsrB and CsrC (Edwards et al., 2011) that sequester and therefore counteract the negative effect of CsrA on protein involved in c-di-GMP and PNAG production, ultimately promoting biofilm formation (see Chapter 3.4.2).

(p)ppGpp has been also described as an important regulator of bacterial virulence. In *S. typhimurium* (p)ppGpp promotes the dimerization of the protein SlyA, which controls the transcription of genes essential for the virulence of this bacterium (Zhao et al., 2008), while in *Francisella tularensis* the binding of the signal molecule to the PigR regulator modulates the expression of virulence genes (Charity et al., 2009).

c-di-GMP. Originally described in 1987 as an allosteric regulator of cellulose synthase complex in *Gluconacetobacter xylinus* (Ross et al., 1987), bis(3'→5')-cyclic dimeric guanylic acid (c-di-GMP) is a bacterial second messenger implicated in the regulation of several processes including biofilm formation, motility, cell cycle, differentiation, and virulence (Römling et al., 2013). The intracellular concentration of c-di-GMP is controlled by diguanylate cyclases (DGC), which contain a GGDEF or GGEEF catalytic domain, and hydrolytic enzymes called c-di-GMP phosphodiesterases (PDE), which contain either an EAL or a HD-GYP domain (Hengge, 2009; Sondermann et al., 2012) (Figure 5). *In vivo* and *in vitro* studies demonstrated that DGC proteins convert two molecules of GTP to cyclic di-GMP (Ryjenkov et al., 2005). Typically DGCs function as homodimers of two GGDEF subunits. The active site (A-site), which binds the cyclic nucleotide, is located at the interface between the two subunits, each binding one of the GTP molecules (Chan et al., 2004; Christen et al., 2005). DGC proteins often contain an inhibitory site (I-site) that is characterized by an RxxD amino acid motif. Binding of c-di-GMP to the I-site allosterically inhibits c-di-GMP synthesis (Christen et al., 2006), helping to avoid excessive GTP consumption and ultimately limiting production of c-di-GMP (Fineran et al., 2007).

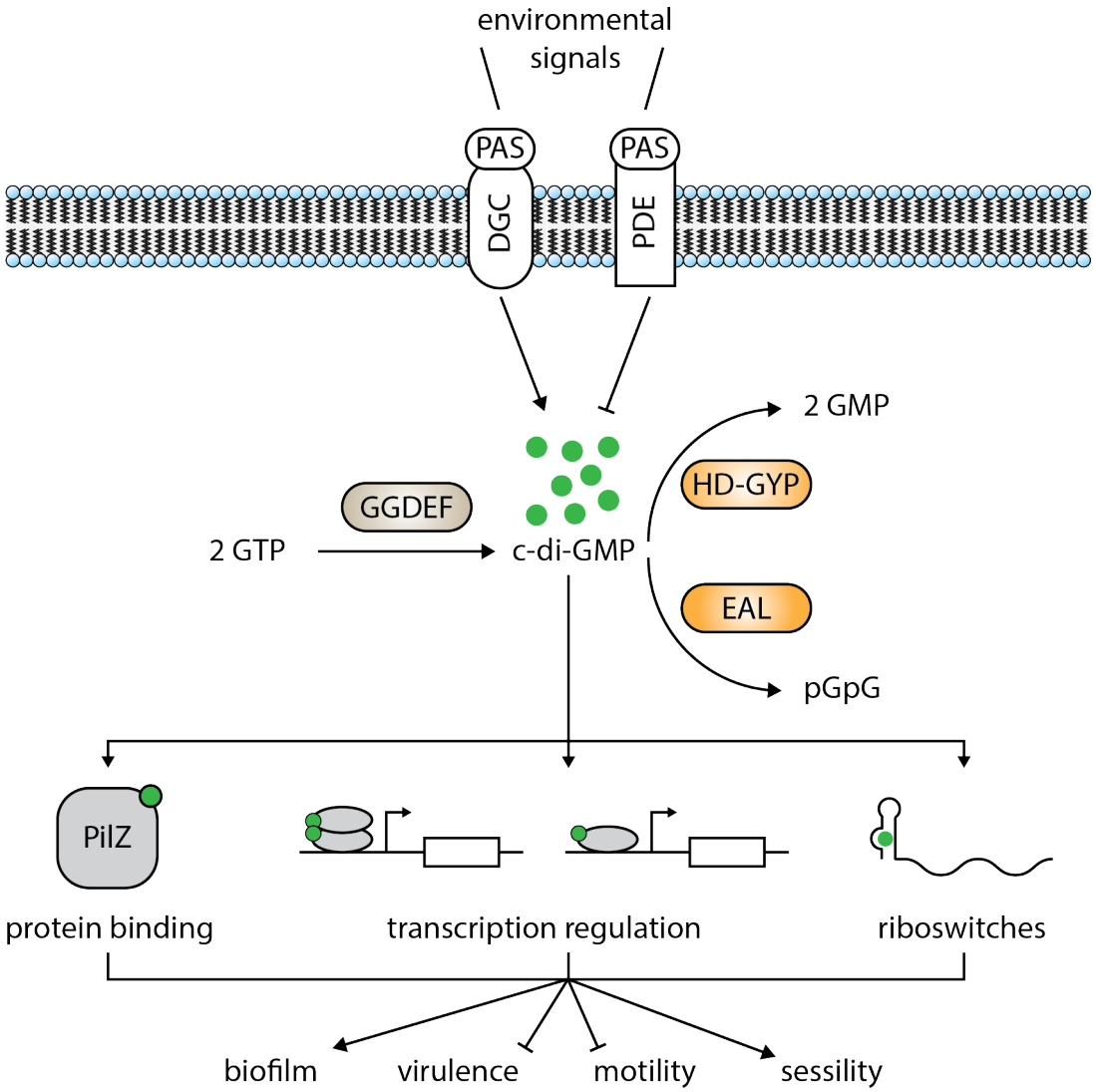

Figure 5. C-di-GMP synthesis, degradation and signaling. Two molecules of GTP are converted to c-di-GMP by DGC proteins containing the GGDEF domain and c-di-GMP is degraded by PDE proteins with EAL or HD-GYP domains. DGCs and PDEs are often found associated with sensor domains such as PAS for sensing gaseous ligands such as O_2,CO_2,NO etc. C-di-GMP binds to receptor proteins such as PilZ, riboswitches or transcriptional regulators to regulate bacterial ‘‘lifestyle’’. Adapted from Kalia et al., 2013

C-di-GMP degradation is carried out by PDE proteins that first hydrolyze the cyclic nucleotide to the linear 5’-phosphoguanylyl-(3’-5’)-guanosine (pGpG) nucleotide and then with a much slower kinetic to two GMP molecules (Schmidt et al., 2005; Christen et al., 2005; Tamayo et al., 2005). In addition to EAL domain, c-di-GMP turnover is regulated by a second class of phosphodiesterases that contain the HD-GYP domain, and can hydrolyze cyclic di-GMP directly in two GMP molecules (Dow et al., 2006; Hengge, 2009; Ryan and Dow, 2010) (Figure 5). Examination of hypothetical proteins from both completed and unfinished genome sequences of a number of

bacteria indicate that HD-GYP proteins are widely conserved, although they are apparently absent from *Escherichia coli* and *Bacillus subtilis* (Slater et al., 2002).

Proteins involved in c-di-GMP metabolism usually contain only one c-di-GMP related domain. However, hybrid proteins containing both GGDEF and EAL domains have been described. Some of them have dual activity, *i.e.* act as both DGC and PDE, depending on cellular state, but usually one of the two domains is inactive (Tarutina et al., 2006; Ferreira et al., 2008; Yang et al., 2011) and can act as regulatory domain by binding to a guanine nucleotide and modulate the activity of the other domain.

Large-scale sequencing of bacterial genomes has revealed that GGDEF and EAL domains are widely distributed, although they are not found in any archeal genome so far (Römling et al., 2005). In general, the number of GGDEF/EAL proteins is much larger in Gram-negative bacteria than in Gram-positive; this may suggest a specialization of Gram-negatives in using this signalling molecules to govern several cellular behaviours (Karatan and Watnick, 2009). The distribution of c-di-GMP among eukaryotes is more complex. The current databases list dozens of GGDEF and EAL domain proteins encoded in plants and lower eukaryotes, such as hydra, sea anemone, *Dictyostelium*, and *Trichoplax*. In any case, no GGDEF/EAL/HD-GYP domain-encoding genes seem to be present in mammals. Moreover, mammalian cells appear to monitor cytoplasmic c-di-GMP, perceived as a sign of bacterial infection, and to launch an innate immune response to counteract the infection (Burdette et al., 2011).

Most DGCs and PDEs couple their GGDEF or EAL domain to sensory domains (PAS, PAC, GAF, BLUF, HAMP and others) that are likely to receive signals from the environment (Figure 5, pag. 32) (Galperin, 2004). PAS are widely conserved domains found in a large number of organisms from bacteria to humans; their activity has been connected with regulation in response to gaseous ligands, redox potential and light (Taylor and Zhulin, 1999). PAC domain usually occurs at the C-terminal part of PAS containing proteins and it is supposed to contribute to the PAS domain folding (Ponting and Aravind, 1997). Structurally similar to PAS, GAF motif is found in cGMP-specific phosphodiesterases, adenylyl cyclases and the bacterial transcription factor FhlA; like PAS domain, GAF is able to bind, either covalently or non-covalently, a remarkably diverse set of regulatory small molecules, many of which are still unknown (Ho et al., 2000). The BLUF domain is usually involved in the repression of photosynthesis genes in response to blue-light. This domain is also found in the DGC YcgF in *E. coli*, a protein that binds to and releases the MerR-like repressor YcgE from its operator DNA upon blu-light irradiation, leading to expression of eight small proteins involved biofilm formation (Tschowri et al., 2009). Furthermore, it has been showed that photoreactivity of the BLUF domain in YcgF protein is dependent on temperature, suggesting that,

in addition to light sensing, the BLUF domain may work as temperature sensor (Nakasone et al., 2010). Finally, HAMP domains are found in bacterial sensor and chemotaxis proteins and in eukaryotic histidine kinases (Pham and Parkinson, 2011; Mondéjar et al., 2012). All these sensory domains together allow bacteria to recognize several environmental and physiological cues, and adjust c-di-GMP levels accordingly. In general, high intracellular c-di-GMP concentration stimulates the synthesis of adhesins and exopolysaccharide-based matrix, whereas it inhibits various form of motility (Figure 5, pag. 32). Hence c-di-GMP levels are generally associated with the transition between the motile planktonic state and the sessile biofilm life-style (Römling et al., 2013).

As already mentioned, c-di-GMP controls several aspects of bacterial life other than biofilm formation. The ability of governing different processes concurrently lies in the diversity of c-di-GMP receptors/interactors, which monitor c-di-GMP levels in the cell and translate it into a specific behavioural response (Mills et al., 2011) (Figure 5, pag. 32). So far, five types of c-di-GMP receptor has been found: effector proteins, such as PilZ domain proteins; degenerated GGDEF/EAL proteins that are no longer catalytically active; proteins with enzymatic activity that bind to c-di-GMP and upon binding, enzymatic proficiency is enhanced; transcription factors or repressors that bind to c-di-GMP to regulate gene expression, and riboswitches that bind to c-di-GMP to regulate transcription or translation.

PilZ domain is commonly found in proteins that do not have any enzymatic activities on their own but bind c-di-GMP to regulate other proteins or enzyme through direct protein-protein interaction. Once PilZ domain bind to c-di-GMP, proteins undergo either structural or aggregation changes, which presumably facilitates the activation of other proteins (Benach et al., 2007). Several PilZ-containing proteins involved in biofilm formation have been characterized so far; for example, this domain is found in the BcsA protein, which is involved in the regulation of cellulose synthesis in *G. xylinus*, S. *typhimurium*, and *E. coli* (Zogaj et al., 2001), in the Alg44 protein that controls alginate production in *P. aeruginosa* (Merighi et al., 2007), in the YcgR protein, which control flagellum rotation in *E. coli* (Paul et al., 2010; Fang and Gomelsky, 2010), and in *Caulobacter crescentus* flagellar motor regulator DgrA (Christen et al., 2007). Another class of non-enzymatic molecules able to bind c-di-GMP is degenerated EAL or GGDEF proteins. Although these proteins have a catalytically inactive reaction centre that shows no *in vitro* activity, they are still able to bind c-di-GMP. A typical example is LapD, an inner membrane protein of *P. fluorescence*. LapD, which contains both DGC and PDE domains, interact with the second messenger using its degenerated EAL motif, and in response to it promotes the maintenance of the specific adhesin LapA on bacteria surface (Newell et al., 2009). Other degenerated GGDEF and EAL domain-containing effectors are

the cell cycle regulator PopA from *C. crescentus* (Duerig et al., 2009), PelD that respond to c-di-GMP level promoting Pel exopolysaccharide production and biofilm formation (V. T. Lee et al., 2007), and FimX from *P. aeruginosa* that is involved in type IV pili assembly and twitching motility (Navarro et al., 2009; Qi et al., 2010).

C-di-GMP can bind directly to and act as an allosteric regulator on proteins with enzymatic activities. Recently, c-di-GMP has been shown to bind to polynucleotide phosphorylase (PNPase) in *E. coli*, affecting its RNA processing and degradation activity (Tuckerman et al., 2011). As already discussed in Chapter 3.4.2, PNPase is an important enzyme involved in RNA degradation that has also a role in PNAG production and therefore biofilm formation (Carzaniga et al., 2012). Although these observations strongly suggest that c-di-GMP can control gene expression at the RNA stability levels through PNPase activity, this effect has not yet been demonstrated.

Several transcription factors respond to environmental signals by binding to second messengers, like CRP that binds cAMP in response to glucose availability (see current Chapter). FleQ has been the first c-di-GMP-dependent transcriptional regulator described. Depending on the level of the second messenger in the cell, FleQ is able to either repress or activate expression of genes involved in PEL polysaccharide production in *P. aeruginosa*. FleQ binds to two distinct sites in the *pel* promoter and interacts with two FleN proteins resulting in a bending of the intervening DNA, and therefore in the repression of the operon transcription. Binding of c-di-GMP relieves the negative effects of FleQ, converting the protein from a repressor to an activator without interfering with interaction with DNA, finally inducing PEL production (Hickman and Harwood, 2008; Baraquet et al., 2012). CLP protein from *X. campestris* is another c-di-GMP-binding transcriptional regulator that shares homology with the *E. coli* cAMP receptor protein (CRP). While most CRP-like proteins bind cAMP, CLP binds c-di-GMP modulating the expression of several genes involved in *Xanthomonas* virulence (Chin et al., 2010). Another example of transcriptional regulator that binds c-di-GMP is VpsT from *V. cholerae*. C-di-GMP binds to VpsT in its dimeric form, affecting motility and biofilm formation in this pathogen (Krasteva et al., 2010).

The last class of c-di-GMP effectors are riboswitches, regulatory motifs found in the UTRs of mRNAs that bind cellular ligands and regulate mRNA transcription or translation, either forming transcription termination stem loop or changing the accessibility of ribosome binding side respectively (Nudler, 2004). Two structurally different c-di-GMP riboswitches has been identified (Sudarsan et al., 2008; Lee et al., 2010). These riboswitches are located upstream of the open reading frame (ORF) of DGC and PDE proteins in some organisms or upstream of some genes controlled by c-di-GMP (Kalia et al., 2013).

As already described for other global regulators, ppGpp, cAMP and c-di-GMP regulatory network are often connected (Kalia et al., 2013), hence further increasing the modulatory capabilities of bacterial regulation in response to environmental and physiological changes. Moreover, c-di-GMP, cAMP and ppGpp are not the only signal molecules that act as global regulators affecting transcription, translation, and protein stability. Other modified nucleotides, such as cGMP, c-di-AMP, has been recently identified and are currently subject of studies (Kalia et al., 2013).

Table 3. Biofilm formation regulation. The table summarizes the signals that regulate biofilm formation with a particular focus on the effects in *E. coli* K-12 strain. Plus and minus signs (+/-) indicate respectively positive or negative regulation; the question mark (?) indicates unknown or not clear effect/regulation.

Signal	Effect on biofilm formation	References	Effect on Escherichia coli K-12		
			Structures/Processes influenced	Regulators	References
Environmental signals					
Glucose	Positive in *S. aureus*, *S. epidermidis* and *V. cholerae*. Negative in *E. coli* K-12	(Dobinsky et al., 2003; Lim et al., 2004; Jackson et al., 2002)	Curli (-) PNAG (+) Flagella (+)	cAMP/CRP, Cra, CsrA, McaS sRNA	(Zheng et al., 2004; Reshamwala and Noronha, 2011; Thomason et al., 2012)
Oxygen	Negative in *P. aeruginosa* Positive in *E. coli* K-12	(Schobert and Tielen, 2010; Landini and Zehnder, 2002)	Curli (+) PNAG (+)	DosCP (c-di-GMP DGC/PDE)	(Tagliabue, Maciag, et al., 2010; Tagliabue, Antoniani, et al., 2010)
Iron	Positive in *P. aeruginosa*, *V. cholerae* and *E. coli* K-12	(Bollinger et al., 2001; Mey et al., 2005)	Curli (+)	BasRS TCS	(Ogasawara et al., 2012)
Phosphate	Negative in *P. fluorescens* Positive in *A .tumefacens* and *E. coli* K-12	(Monds et al., 2007; Danhorn et al., 2004)	Curli (+) RpoS-dep. structures (+)	RpoS stabilized by IraP	(Bougdour et al., 2006)
Magnesium	Positive in *E. coli* K-12	-	Curli (+)	PhoPQ/RstAB TCSs	(Ogasawara et al., 2007)
Sulphate	Positive in *E. coli* K-12	-	Curli (+)	?	Rossi and Landini (see Part III)
Osmolarity	Positive/Negative in *E. coli* K-12		Curli (-) PNAG (+)	EnvZ/OmpR TCS, OmrA/OmrB sRNAs, CpxAR TCS, H-NS	(Jubelin et al., 2005; Goller et al., 2006; Vianney et al., 2005; Holmqvist et al., 2010)
Temperature (≥ 37°C)	Positive in pathogenic *E. coli* strains Negative in *E. coli* K-12	(Olsén et al., 1993; Cookson et al., 2002)	Curli (-)	Crl, DsrA sRNA, c-di-GMP	(Bougdour et al., 2004; Repoila and Gottesman, 2001; Navarro et al., 2009)

Signal	Effect on biofilm formation	References	Effect on Escherichia coli K-12		
			Structures/Processes influenced	Regulators	References
Host-derived signals					
Bile	Positive in *V. cholerae* and *Lactobacillus* strains	(Hung et al., 2006; Ambalam et al., 2012)	-	-	-
Hydrogen peroxide (H2O2)	Positive in *P. aeruginosa*	(Mathee et al., 1999)	-	-	-
Physiological and metabolic signals					
Indole	Positive *K. oxytoca*, *P. stuartii*, *C. koseri*, *M. morganii*, *H. influenza* and *V. cholerae* Positive/Negative in *E. coli* K-12 (?)	(Martino et al., 2003; Mueller et al., 2007)	Motility (-)	SdiA	(Lee et al., 2007)
D-amino acids	Negative in *B. subtilis* and other Gram-negative and Gram-positive bacteria	(Kolodkin-Gal et al., 2010)	-	-	-
Polyamines	Positive (Spermidine)/Negative (Norspermidine) in *V. cholerae* Positive in *E. coli* K-12 and *Y. pestis* Negative in *B. subtilis*	(Karatan et al., 2005; McGinnis et al., 2009; Wortham et al., 2010; Kolodkin-Gal et al., 2012)	?	PotD	(Zhang et al., 2013)

Signal	Effect on biofilm formation	References	Effect on Escherichia coli K-12		
			Structures/Processes influenced	Regulators	References
Cellular density	Positive in *P. aeruginosa* and *E. coli* K-12	(Davies et al., 1998; Sakuragi and Kolter, 2007; Ueda and Wood, 2009)	Ag43 (+) Motility (+) Colanic acid (-)	MqsR/YncC/YbiM, DsrA	(González Barrios et al., 2006; Li et al., 2007; Zhang et al., 2008)
Slow growth/Stationary phase	Positive/Negative in *P. aeruginosa*. *Positive in E. coli K-12*	(Whiteley et al., 2000; Schembri et al., 2003)	Type I fimbrae (-) Curli (+) Cellulose (+)	RpoS, MlrA, YciR/YegG/YdaM/YciR (c-di-GMP level)	(Arnqvist et al., 1994; Dove et al., 1997; Lindenberg et al., 2013)
Envelope stress	Negative in *E. coli* K-12	-	Colanic Acid (+) Flagellum (-) Curli (-)	RcsCD/RcsB, RprA sRNA	(Vianney et al., 2005; Majdalani and Gottesman, 2005; Mika et al., 2012)
Nucleotide pool, UMP, Uracil	Positive in *E. coli* K-12	-	Curli (+) Cellulose (+/-)	?	(Garavaglia et al., 2012)

4 Curli fibres

Curli fibres, or thin aggregative fimbriae (Tafi), are flexible proteinaceous fibrils made up of a major subunit (CsgA) and a nucleator protein (CsgB). These extracellular structures are typical of enterobacteria, such as *Escherichia coli* and *Salmonella* spp. (Olsén et al., 1989; Collinson et al., 1991; Römling, Bian, et al., 1998); in these bacteria curli are key factors that mediate surface adhesion, cell-to-cell aggregation (Cookson et al., 2002; Vidal et al., 1998), and with cellulose and other exopolysaccharides constitute the biofilm extracellular matrix (Hung et al., 2013; Serra et al., 2013). In addition, curli fibres produced by enteroinvasive (EIEC) and enterohaemorrhagic (EHEC) *E. coli* strains seem to be important during infection and epithelial cell invasion (Sukupolvi et al., 1997; Gophna et al., 2001; Cookson et al., 2002; Uhlich et al., 2002). Although curli have been considered a characterizing feature of Gram-negative enterobacteria biofilms, recently similar structures have been identified in the Gram-positive bacteria *Mycobacterium tuberculosis* (Alteri et al., 2007) and *Bacillus subtilis* (Romero et al., 2010). Furthermore, analogous protein complexes have been identified as the major proteinaceous component of many biofilms in several environmental isolates belonging to *Gammaproteobacteria*, *Bacteriodetes*, *Firmicutes* and *Actinobacteria* (Larsen et al., 2007).

4.1 Curli biogenesis

Gene coding for curli proteins are organized in two divergent operons, *csgBAC* and *csgDEFG* that are separated by 513 bp, constituting one of the most wide intergenic region in *E. coli* (Hammar et al., 1995) (Figure 6, pag. 38). Homologous operons have been identified in *Salmonella* spp. and are called *agfBA* and *agfDEFG* (Römling, Bian, et al., 1998).

The *csgBAC* operon encodes the major structural subunit, CsgA, and the nucleator protein CsgB (Hammar et al., 1995, 1996). Although a rapidly growing number of bacterial species are now known to produce amyloid fibers, the third gene of the *csgBAC* operon, *csgC*, is found exclusively within a group of closely-related *Enterobacteriaceae* including *Escherichia* and *Salmonella* (Taylor et al., 2011). In *E. coli,* CsgC has been considered not relevant to curli biogenesis until recently (Barnhart and Chapman, 2006). Indeed, mutant strains lacking *csgC* still assemble curli yet they show defects in auto-aggregation, form paler colonies on Congo red plates, and display variable affinity for soluble fibronectin (Hammar et al., 1995). A recent study has suggested that CsgC is related to the N-terminal domain of the DsbD oxido-reductase, both in structure and oxido-

reductase capability. CsgC seems to act on the redox state of a cysteine residue present in the CsgG curli exporter protein, changing CsgA and CsgB flux through the CsgG pore to ultimately regulate the amount of curli synthesized (Taylor et al., 2011).

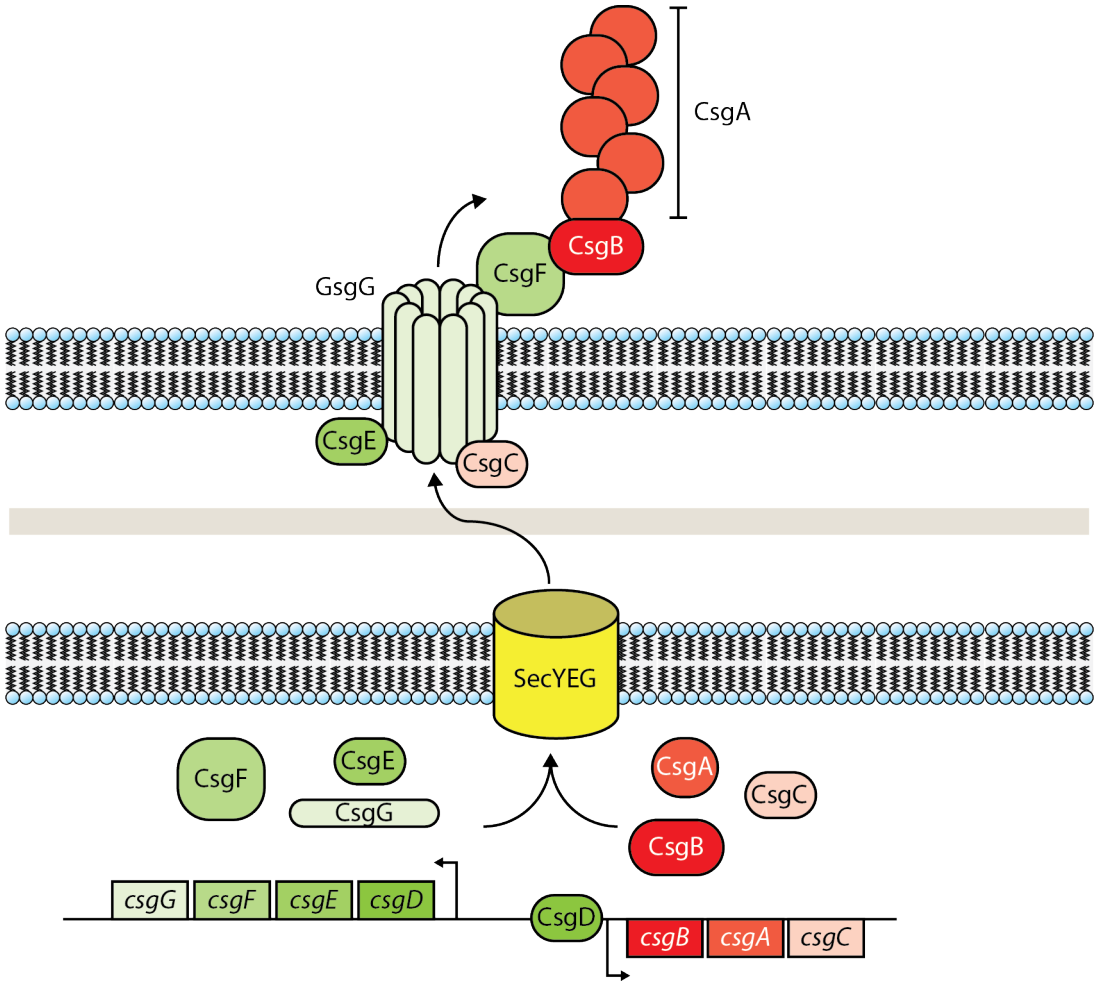

Figure 6. Model of curli biogenesis. Except CsgD, all the proteins contain a sec signal sequence necessary for translocation to periplasm. Curli major subunit CsgA and curli minor subunit CsgB are secreted through the CsgG-formed pore with the help of CsgE and CsgF proteins. In this model CsgC interacts with CsgG channel regulatin the flux of CsgA and CsgB proteins. On the cell surface CsgB-mediate nucleation of CsgA occurs in the proximity of CsgG complex. Adapted from Blanco et al., 2012.

The *csgDEFG* operon encodes the curli master regulator CsgD and three accessory protein CsgE, CsgF, CsgG necessary for curli assembly (Hammar et al., 1995). CsgG is an outer membrane (OM) lipoprotein and is required for the stability and secretion of CsgA and CsgB (Loferer et al., 1997; Chapman et al., 2002). CsgE is a periplasmic protein; mutants lacking this gene are defective in curli assembly, although they are still able to produce a reduced amount of CsgA fibres that however show a distinct morphology from those produced by wild-type cells (Chapman et al.,

2002). CsgE also physically interacts with CsgG at the outer membrane (Robinson et al., 2006). Finally, CsgF is a periplasmic protein that also interact with CsgG in the OM, and its inactivation prevent CsgA polymerization at the membrane surface (Chapman et al., 2002; Robinson et al., 2006).

Curli proteins, after their synthesis in the cytoplasm, are secreted into the periplasm by the Sec-translocation system. Once in the periplasm, the lipoprotein CsgG is transported by the LOL (lipoprotein outer-membrane localization) transport system to the OM where it oligomerizes into a pore-like structure that is required for secretion in the extracellular space of the major subunit CsgA and the minor subunit CsgB (Robinson et al., 2006; Epstein et al., 2009). At the cell surface, CsgA is nucleated into a fibril by cell-surface-associated CsgB. CsgA nucleation occurs at specific locations on the surface that are dictated by CsgG clustering (Hammer et al., 2007; Epstein et al., 2009). During the assembly process CsgE and CsgF assist curli subunit transit through the periplasm acting as chaperons, while CsgC, as already mentioned, influences the transport rate of curlin through CsgG pore (Chapman et al., 2002; Taylor et al., 2011) (Figure 6, pag. 38).

Curli subunits assemble into fibres that display amyloid properties. From bacteria to humans, amyloid fibres share the ability to fold into a characteristic cross-β-strand structure, where the β-sheets are oriented perpendicular to the axis of the fibres. Amyloid fibres formation is traditionally associated with degenerative protein-folding events that lead to severe human diseases such as Alzheimer, Parkinson (Cohen and Kelly, 2003); however, curli are the product of a tightly regulated and directed process, and are part of a growing number of functional amyloids that have now been identified in all kingdoms of life from bacteria to humans (Fowler et al., 2007). Like other amyloid fibres, curli bind to Congo red dye conferring a typical red colour to bacterial colonies that synthetize these structures (Klunk et al., 1989).

4.2 Curli expression regulation

Curli fibres represent the major biofilm determinant in *Escherichia coli* and therefore their expression is regulated in response to a surprisingly high number of environmental and physiological stimuli, resulting in one of the most rich and layered regulatory networks known so far in bacteria (Figure 7, pag. 40). Production of curli fibres is controlled both at transcriptional and post-transcriptional level through the concerted action of several different effectors including transcriptional regulators, non-coding RNAs, and small molecules. Furthermore, although curli

regulation has been extensively studied in the last twenty years, several different regulatory circuits remain still unknown.

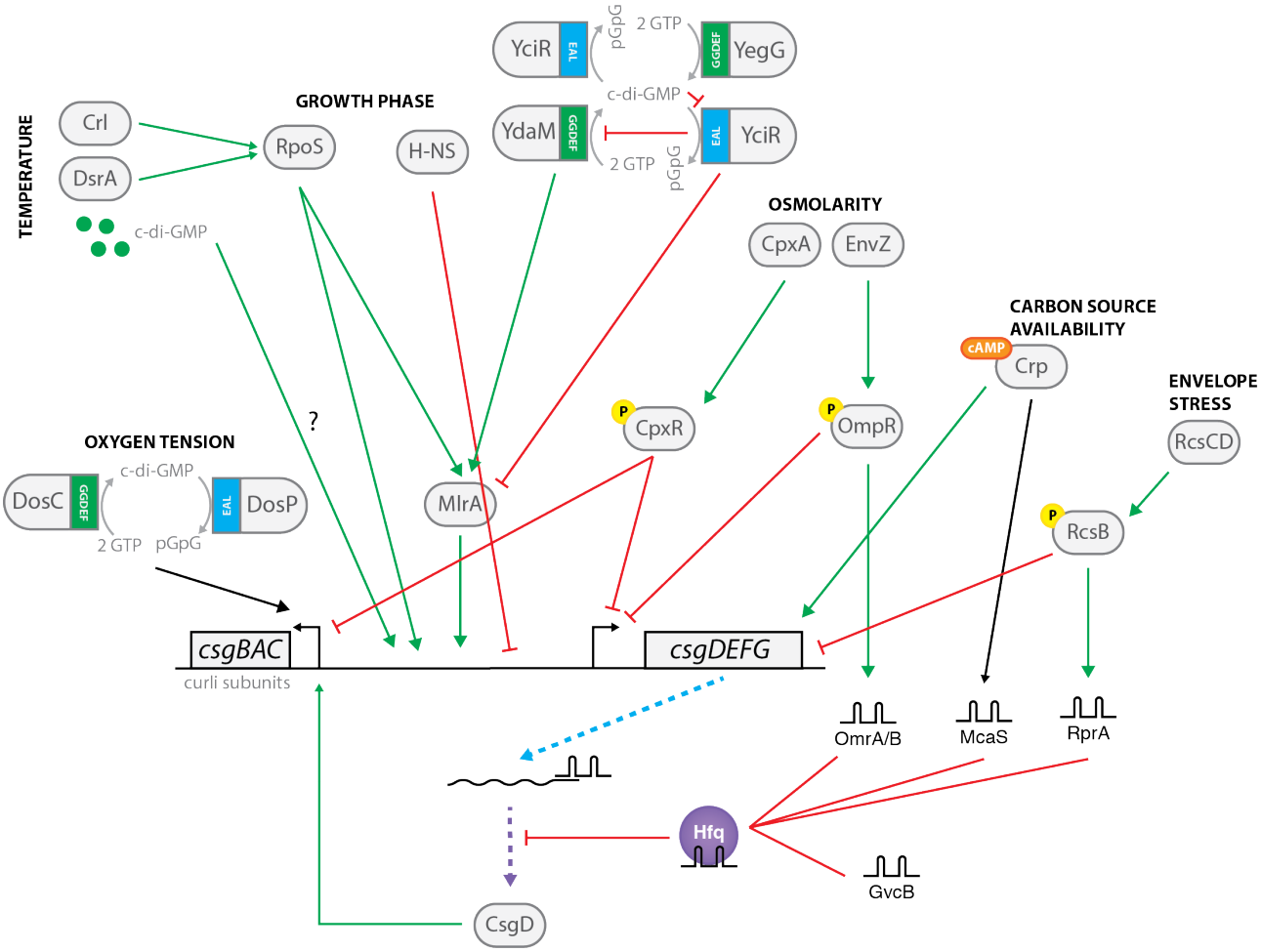

Figure 7. Schematic representation of the complex network that regulates curli expression. In the figure are represented only a selected part of the known regulators, other transcriptional regulators have been omitted.

4.2.1 Transcriptional regulation

The transcriptional regulator CsgD plays a central role in curli regulatory network. CsgD is part of the LuxR superfamily, which is characterized by a conserved N-terminal activation domain and a conserved C-terminal helix-turn-helix (HTH) DNA binding domain. Based on the homology of the N-terminal activation domain CsgD is classified as a member of the FixJ/UhpA subfamily of regulators. FixJ family members are typically response regulator in prokaryotic two-component systems and are subjected to phosphorylation at a specific aspartate residue at position 58 by a sensor kinase. Although the phosphorylation target Asp58 is present in the receiver domain of CsgD protein, only five amino acids involved in this modification are conserved (Römling et al., 2005).

Thus, while phosphorylation of Asp58 has been proposed to affect stability and DNA binding capabilities of CsgD, the mode of its activation remains unknown and a cognate sensor kinase has not been identified (Zakikhany et al., 2010).

CsgD binds to a putative conserved 11-bp sequence upstream the *csgBAC* and *csgDEFG* operons controlling curli expression (Römling, Sierralta, et al., 1998; Zakikhany et al., 2010; Ogasawara et al., 2011). Beside curli, CsgD co-regulate the production of cellulose, the second major component of *E. coli* biofilm matrix, acting on the transcription of the *adrA* gene, which codes for a diguanylate cyclase required for cellulose synthase complex activation (Römling et al., 2000). Furthermore, CsgD expression has a broader effect on the cell causing an indirect genome-wide up- and down-regulation of transcription of several genes probably creating a physiological condition favourable for biofilm determinants production (Römling, 2005).

Several different environmental and physiological conditions affect CsgD transcription and therefore curli production: growth rate, temperature, osmolarity, oxygen tension, pH, ethanol exposition, and nutrients availability have been all identified as important regulators of curli expression.

Growth rate is arguably the most important factor for curli production. Curli operons are normally expressed during slow growth conditions such as stationary phase and in nutrient-limited conditions, *i.e.* minimal media (Arnqvist et al., 1994). Generally speaking, a wide number of genes is affected by slow metabolism, and their transcription in these conditions often depends on the alternative sigma factor RpoS (see Chapter 3.4.1) (Lacour and Landini, 2004; Maciag et al., 2011; Landini et al., 2013). In stationary phase RpoS accumulates in cells competing with the housekeeping σ^{70} factor for the binding with core RNAP. Both sigma factors are able to recognize *csg* promoters, but during exponential growth σ^{70}-dependent expression is hindered by the "exponential silencing" mechanism that relies on H-NS repression. However, RpoS is able to overcome H-NS negative effects leading to curli genes expression (Arnqvist et al., 1994). Hence, *csgDEFG* and *csgBAC* operons are effectively transcribed only during specific conditions that promote σ^{S} expression and accumulation. Furthermore, RpoS accumulation during stationary phase induces the expression of the MlrA protein, which binds and modifies the conformation of *csgD* promoter fostering the transcriptional activation of *csg* genes through the RNAP-RpoS complex (Brown et al., 2001; Ogasawara et al., 2010).

Almost all *S. typhimurium* and *S. enteritidis* strains from human animals and food as well as other clinical isolates and *E. coli* K-12 strains express curli fibres at temperature lower than 32°C, but not at 37°C (Gerstel and Römling, 2003). However, this regulation is not present in other pathogenic enterobacteria that express this kind of fimbrae at 37°C during host invasion and colonization (Bian

et al., 2000). Expression analysis revealed that temperature regulation takes place at the level of *csgD* transcription, and point mutations in the *csgD* promoter determine the loss of temperature-dependent expression (Römling, Sierralta, et al., 1998).

The mechanism governing curli temperature-dependent expression is still unknown. However, it has been proposed that two different effectors acting as thermo sensors may be involved in such regulation. The first effector is the Crl protein; Crl expression is increased during the transition from growing to stationary phase and it accumulates only at low temperature (30°C). Crl is able to directly bind to RpoS sigma factor promoting its association with RNAP core (Typas et al., 2007) and increasing its affinity for *csgBAC* promoter, therefore promoting curli expression (Bougdour et al., 2004). However, some strains lacking the Crl protein are still able to synthesize curli fibres suggesting that Crl can contribute to temperature-dependent regulation only in some conditions (Provence and Curtiss, 1992). The second regulatory system depends on the small regulatory RNA DsrA. DsrA is heavily transcribed and stabilized at low temperatures, and once bound to RpoS mRNA it promotes translation of sigma factor's messenger (Repoila and Gottesman, 2001). A third possible contribution to temperature regulation could depend on the second messenger c-di-GMP. Curli synthesis is indeed stimulated upon c-di-GMP accumulation (see Chapter 4.2.3). It has been proposed the amount of enzymes involved in the synthesis of this modified nucleotide is higher at low temperature (Sommerfeldt et al., 2009), suggesting therefore that c-di-GMP could accumulate at low temperature contributing to temperature-dependent expression of curli fibres.

Osmolarity is another key regulator of curli expression. In particular, low osmolarity condition stimulates the transcription of *csgDEFG* operon (Vidal et al., 1998). This effect is dependent on the response regulator OmpR, which, in its phosphorylated form, binds upstream to the -35 region of *csgD* promoter activating its transcription (Prigent-Combaret et al., 2001). A second independent mechanism acts during high osmolarity repressing curli production through the activity of the CpxA/CpxR TCRS and the global regulator H-NS (Dorel et al., 1999; Jubelin et al., 2005).

It's interesting to note that activation of the two systems depends on the osmolyte nature. In the presence of high saline concentrations, phosphorylated CpxR (CpxR-P) accumulates inside the cell and binds several different target sequences in the promoters of both curli operons, repressing their transcription in response to the increasing osmolarity. On the contrary, when the environment is saturated by an elevated concentration of sugars the repression depends exclusively on the activity of H-NS (Vianney et al., 2005).

In *S. typhimurium*, low oxygen condition stimulates *csgD* transcription, suggesting that regulation of the *csgDEFG* operon is also dependent on environmental oxygen tension (Gerstel and Römling, 2001). In *E. coli*, curli oxygen regulation seems to be mediated by c-di-GMP and by the activity of

DosC and DosP proteins, a DGC and a PDE that together control the second messenger concentration (Sondermann et al., 2012). Both proteins have a heme protestetic group that binds O_2, CO, and nitrogen oxide (NO), and together form a stable complex (Delgado-Nixon et al., 2000; Tuckerman et al., 2009). Interestingly, it has been shown that DosC/P complex activity affects the production of curli fibres acting only on the transcription of the structural operon *csgBAC* without inducing the expression of the regulatory operon *csgDEFG* (Tagliabue, Maciag, et al., 2010).
In addition to the regulatory circuits described so far, several other regulators influence curli transcription. The Rcs phosphorelay system is involved in membrane maintenance, colanic acid and flagella production (Majdalani and Gottesman, 2005). Furthermore, Rcs system is implicated in the negative regulation of the two *csg* operons in a RcsA-dependent manner (Vianney et al., 2005). Another mechanism governing curli production is represented by the PhoQP and RstBA TCRSs. At low Mg^{2+} concentration the sensory protein PhoQ phosphorylates the response regulator PhoP, which positively regulates the expression of *rstB* and *rstA* genes. The two genes code for a second two-component response system in which RstB is the sensor and RstA the response regulator. Experimental data suggest that RstA may bind *csgD* promoter in a region overlapping an OmpR target sequence, hence working as a repressor of curli expression. Thus, low Mg^{2+} concentration in the environment inhibits curli transcription (Ogasawara et al., 2007).

4.2.2 Post-transcriptional regulation

CsgD mRNA has been recently described as a hub for environmental and physiological signal integration via multiple small RNAs. Indeed, five different sRNAs has been shown to interact with and regulate *csgD* messenger translation and stability (Boehm and Vogel, 2012) (Figure 7, pag. 40). OmrA/OmrB sRNAs are regulated through EnvZ/OmpR TCRS in response to osmolarity and are involved in reshaping the composition of outer membrane proteins, negatively regulating the levels of *ompT, cirA*, *fecA*, *fepA* (Guillier and Gottesman, 2006), *ompR* (Guillier and Gottesman, 2008), and the flagellar regulator *flhDC* (De Lay and Gottesman, 2012). Moreover, OmrA/B directly regulate *csgD* expression by a direct antisense interaction within the *csgD* 5'-UTR, far upstream of the ribosome-binding site (RBS); this interaction requires the activity of the RNA chaperone Hfq (Holmqvist et al., 2010). Similar to OmrA/B sRNAs, other three regulators (McaS, RprA, and GcvB) act as repressor by base paring with the 5'-UTR of the *csgD* mRNA. Each of them belongs to a different regulon and is expressed under different conditions. McaS is regulated by the CRP/cAMP system, and therefore its levels in cell respond to carbon source availability. In addition

to repress CsgD expression (Thomason et al., 2012; Jørgensen et al., 2012), McsA stimulates the production of the flagellar master regulator FlhDC and of the PNAG polysaccharide (Thomason et al., 2012). Therefore, one can speculate that McsA promotes the formation of a biofilm whose extracellular matrix is composed by the extracelluar polysaccharide PNAG and require flagella contribution, which ultimately differs from the one achieved through the activity of the regulator CsgD (curli/cellulose matrix).

RprA sRNA is activated by the Rcs system upon envelope stress and, it exerts both a negative and positive role on curli. Indeed, RprA negatively regulates curli expression either directly by binding to *csgD* mRNA, or indirectly hampering the synthesis of the diguanylate cyclase YdaM, a protein required for a robust transcription of *csg* operons (see Chapter 4.2.3). On the contrary, the same sRNA activates the synthesis of the global regulator RpoS, which stimulates CsgD and curli expression (Mika et al., 2012).

The last sRNA, GcvB, is regulated in response to the amino acids availability via the transcription factors GcvR and GcvA of the glycine cleavage system (Urbanowski et al., 2000). In addition to controlling the synthesis of different amino acids transporters and the two component system PhoQ/PhoP (Coornaert et al., 2013), GcvB is directly implicated in the negative regulation of *csgD* mRNA expression (Jørgensen et al., 2012).

This evidence suggests that signal integration and gene regulation acting at the *csgD* mRNA level might be equally sophisticated as its transcription-factor based counterpart acting at the DNA level, with the long 5'-UTR of *csgD* mRNA playing a similar role to the complex promoter of *csg* operons.

4.2.3 Second messenger-dependent regulation

Two second messenger molecules, cAMP and c-di-GMP, directly regulate the expression of *csgDEFG* and *csgBAC* operons.

Intracellular cAMP levels depend on environmental glucose concentration and are monitored through the CRP transcriptional regulator (see Chapter 3.4.4). The *csgDEFG* operon belongs to the CRP regulon (Zheng et al., 2004), and the cAMP-CRP complex recognizes at least two different binding sites in the *csgD* promoter positively regulating its expression. Thus, at low glucose concentration, cAMP synthesis is high and curli production reaches its maximum. In addition, as already described in the previous chapter, cAMP-CRP also stimulates the production of the McaS sRNA, which negatively affects *csgD* expression and stimulates flagella and PNAG production (Thomason et al., 2012). Consequently, cAMP exert both a positive and negative role on curli

production: it stimulates transcription of curli operons, while inhibiting translation of *csgDEFG* mRNA. This regulative loop could therefore play an important role in fine tuning the expression of the CsgD master regulator in response to the ever-changing glucose level, thus allowing the cell to modulate the production of an extracellular matrix that is not only constituted by curli and cellulose, but also of other proteins and polysaccharides.

C-di-GMP second messenger mediates the transition between planktonic and biofilm life-styles (see Chapter 3.4.4). Therefore, it comes as no surprise that c-di-GMP levels strongly affect curli genes transcription (Pesavento et al., 2008). The minimal 'module' required for c-di-GMP signalling consists of a DGC, a PDE and an effector component that directly controls the output of a specific target (Hengge, 2009). Three different c-di-GMP signalling modules have been connected with the regulation of curli operons: DosC/DosP, YdaM/YciR and YhjH/YegE. YegE/YhjH and YdaM/YciR modules operate together as a signalling cascade in which c-di-GMP controlled by the YegE/YhjH DGC/PDE pair regulates the activity of the YdaM/YciR pair. In particular, YegE/YhjH generates c-di-GMP when cells approach stationary phase and prevents the PDE YciR from inhibiting the DGC YdaM; thus, YdaM is free to generate c-di-GMP and through direct interaction with the transcriptional regulator MlrA to activate *csgD* transcription. Thus, it was suggested that these two modules respond on a local self-controlled concentration of c-di-GMP, rather than be influenced by the global second messenger levels (Lindenberg et al., 2013). These pieces of evidence offer a clear view on how several different DGC/PDE modules that act all together on concentration of a same freely diffusible molecule can regulate only a specific cellular behaviour.

As already described before, the DosC/DosP pair controls the transcription of the sole *csgBAC* operon without affecting the *csgDEFG* operon in response to oxygen tension (Tagliabue, Maciag, et al., 2010). Although the effector component of the DosC/DosP system has not been identified, it might be tempting to speculate that the Dos system might affect *csgBAC* RNA levels through regulation of the RNA degradosome: indeed, the DosCP complex copurifies with the degradosome and might interact directly with PNPase, whose RNA-degrading activity is affected by c-di-GMP (Tuckerman et al., 2011). Thus, c-di-GMP synthetized in response to oxygen may ultimately affect the processing of one or more sRNAs that post-transcriptionally regulate *csgD* mRNA translation. This mechanism should lead only to the transcriptional induction of the *csgBAC* operon without affecting transcript levels of the *csgDEFG* operon, in agreement with the results obtained by Tagliabue and co-workers (Tagliabue, Maciag, et al., 2010).

4.2.4 Metabolic products

The curli-encoding operons represent a main feature in the control of biofilm development in *E. coli*. Indeed, the CsgD protein controls the expression of the two major constituent of the biofilm matrix in this bacterium: it activates *csgBAC*, leading to curli biosynthesis, and it stimulates the expression of *adrA*, which encodes a diguanylate cyclase required for cellulose synthase activation (Römling, Sierralta, et al., 1998). The transition to biofilm cells, triggered by curli fibres and cellulose, requires considerable energy production. Thus, the metabolic state of the cell has a great impact on the regulation of the two *csg* operons. Recently, several different metabolic pathways and metabolites have been shown to directly affect *csgBAC* and *csgDEFG* transcription. Catabolite repressor activator (Cra), also known as fructose repressor (FruR), is a transcriptional regulator that plays a pleiotropic role to modulate the direction of carbon flow through different metabolic pathways of energy metabolism in a cAMP/CRP-independent manner (Saier, 1996). As curli biosynthesis operons are highly repressed in *cra* deficient strains, it was hypothesized that Cra may directly regulate the expression of these operons. Indeed, four putative Cra binding sites have been identified in the curli intergenic region and direct protein binding to the *csgD* promoter has been proved (Reshamwala and Noronha, 2011). In presence of glucose, D-fructose 1-phosphate and D-fructose 1,6-phosphate metabolites accumulate in the cell, and prevent Cra from binding target DNA and to activate curli genes. Thus, environmental levels of glucose repress curli production through the cAMP/CRP regulon, while the Cra protein negatively regulates the two *csg* operon in response to two glycolysis intermediates (D-fructose 1-phosphate and D-fructose 1,6-phosphate), whose concentration directly depends on the intracellular glucose availability. These represent an interesting phenomenon, which is found also in other regulative processes: cell regulates the production of extracellular structures sensing both the intracellular and extracellular concentration of the same molecule, respectively monitoring its metabolism or by exploiting a system that directly senses its concentration. Thus, depending on its origins, the same molecule can simultaneously regulate different aspect of bacterial behaviour.

Curli expression is also regulated in response to intracellular concentration of *N*-acetylglucosamine-6-phosphate (GlcNAc-6P), an important intermediate in peptidoglycan, lipd IV and enterocommon antigen (ECM) biosynthesis. Although the molecular mechanism is still unknown, transcription of curli operons in mutants that accumulates the metabolic intermediated is repressed, suggesting that the acetylated sugar could act as a negative determinant in curli biosynthesis (Barnhart et al., 2006).

Published works from the laboratory in which I have worked during my Ph.D. have tackled the problem of how different metabolic pathways can regulate curli fibres and other extracellular structures ultimately affecting the biofilm formation process. As detailed in the following chapters, the manuscript Garavaglia et al., 2012, "The Pyrimidine Nucleotide Biosynthetic Pathway Modulates Production of Biofilm Determinants *in Escherichia coli*" (see Part II), shows that curli and cellulose production is tightly linked to nucleotide biosynthetic pathways. As suggested by mutants unable to synthesize UMP, low intracellular concentrations of pyrimidines seem to prevent transcription of curli-encoding operons *csgBAC* and *csgDEFG*. On the contrary, increased *de novo* nucleotide synthesis seems to stimulate expression from the two transcriptional units; furthermore, we demonstrated that the presence of exogenous uracil could trigger cellulose production activating the diguanylate cyclase YedQ. Our observations suggest that production of cellulose and curli, usually co-regulated, can be unbalanced depending on the relative activities of the *de novo* versus the salvage pathways for UMP biosynthesis. More in general, we also showed that nucleotide pool disruption, obtained blocking both pyrimidine and purine nucleotide synthesis, can negatively affect curli fibres production. Thus, since curli and cellulose are pivotal for biofilm formation, nucleotide biosynthesis could be a good candidate for drugs endowed with anti-biofilm activity. Indeed, in the attached manuscript Antoniani et al., 2013, "The immunosuppressive drug azathioprine inhibits biosynthesis of the bacterial signal molecule cyclic-di-GMP by interfering with intracellular nucleotide pool availability" (see Part II), we showed that the drug azathioprine, an inhibitor of 5'-aminoimidazole-4-carboxamide ribotide (AICAR) transformylase, an enzyme involved in purine biosynthesis, could prevent biofilm formation in clinical isolates of *E. coli*, through the reduction of the nucleotide pools available for the synthesis of the second messenger c-di-GMP.

Finally, in the attached manuscript Rossi and Landini, "Phosphoadenosine 5'-phosphosulphate (PAPS) levels affect production of curli fibres and other extracellular factors in *Escherichia coli*" (see Part III), we showed that production of curli fibres is also affected by yet another biosynthetic pathway, namely, sulphate reduction for cysteine/methionine biosynthesis. Indeed, curli proteins synthesis is higher in cells unable to convert the pathway intermediate phosphoadenosine 5'-phosphosulfate (PAPS) to sulphite and adenosine 3',5'-bisphosphate (pAp); this effect seems to be mediated by the stabilization of the sole *csgBAC* mRNA, while the second curli encoding operon *csgDEFG* is not affected. Furthermore, no other intermediate of the cysteine biosynthetic pathway shows a similar effect, thus suggesting that is the modified nucleotide PAPS that may indirectly influence curli production, acting on the *csgBAC* operon, and therfore modulating either the

availability of the structural subunits (CsgAB) or their export rate (through the CsgC protein) in response to environmental sulphur concentrations.

In conclusion, all the data presented in this thesis and partly already published, clearly suggest that cellular metabolism and small metabolic molecules, which reflect the environmental condition in which bacteria are growing, play a pivotal role in regulating the processes of biofilm formation, acting directly on the production of biofilm determinants. The creation of local microenvironments during biofilm formation affects the metabolism of cell subpopulations, changing the expression of specific extracellular structures and creating a heterogeneous community. Thus, one can speculate that the reshaping of the cellular surface in response to the changing surroundings leads to the formation of a dynamic biofilm structure, continuously remodelled to adapt to the current milieu, a completely different structure from the static one described by the current biofilm development model. The results collected in this Ph.D. thesis strongly support the notion that metabolic fluxes of different essential elements, such as sulphur, play a crucial role in controlling cell surface reorganization. Accumulation of intermediate metabolites is instrumental in relaying to the bacterial cell conditions of lack or abundance of a given element, triggering the adequate responses. The molecular mechanisms involved in this process seem to be extremely complex and probably involve gene regulation control at transcription initiation and RNA and protein stability.

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PART II

Content

Research article 1:

Garavaglia M, **Rossi E,** Landini P. 2012. The Pyrimidine Nucleotide Biosynthetic Pathway Modulates Production of Biofilm Determinants in Escherichia coli. PLoS One **7**:e31252.

Research article 2:

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The Pyrimidine Nucleotide Biosynthetic Pathway Modulates Production of Biofilm Determinants in *Escherichia coli*

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Abstract

Bacteria are often found in multicellular communities known as biofilms, which constitute a resistance form against environmental stresses. Extracellular adhesion and cell aggregation factors, responsible for bacterial biofilm formation and maintenance, are tightly regulated in response to physiological and environmental cues. We show that, in *Escherichia coli*, inactivation of genes belonging to the *de novo* uridine monophosphate (UMP) biosynthetic pathway impairs production of curli fibers and cellulose, important components of the bacterial biofilm matrix, by inhibiting transcription of the *csgDEFG* operon, thus preventing production of the biofilm master regulator CsgD protein. Supplementing growth media with exogenous uracil, which can be converted to UMP through the pyrimidine nucleotide salvage pathway, restores *csgDEFG* transcription and curli production. In addition, however, exogenous uracil triggers cellulose production, particularly in strains defective in either *carB* or *pyrB* genes, which encode enzymes catalyzing the first steps of *de novo* UMP biosynthesis. Our results indicate the existence of tight and complex links between pyrimidine metabolism and curli/cellulose production: transcription of the *csgDEFG* operon responds to pyrimidine nucleotide availability, while cellulose production is triggered by exogenous uracil in the absence of active *de novo* UMP biosynthesis. We speculate that perturbations in the UMP biosynthetic pathways allow the bacterial cell to sense signals such as starvation, nucleic acids degradation, and availability of exogenous pyrimidines, and to adapt the production of the extracellular matrix to the changing environmental conditions.

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Introduction

Bacteria are able to switch between two different "lifestyles": single planktonic cells and sessile microbial communities, or biofilms. Biofilm cells are characterized by production of adhesion factors and extracellular polysaccharides (EPS) constituting the so-called "biofilm matrix" that, in addition to promoting cell-cell aggregation and cell-surface adhesion, can confer bacterial cell resistance to various environmental stresses [1–4]. Transition from planktonic cells to biofilm, as well as biofilm maturation and dispersal, responds to environmental and physiological cues, usually relayed to the bacterial cell by signal molecules. Accumulation of signal molecules triggers biofilm formation and maintenance by stimulating the production of adhesion factors, either by activating transcription of corresponding genes or by increasing activity of EPS biosynthetic enzymes. In Gram negative bacteria, the modified nucleotide cyclic-di-GMP (c-di-GMP) plays a pivotal role in biofilm formation and maintenance by stimulating production of EPS and adhesion factors [5–8], while negatively affecting cell motility [9,10]. Another class of signal molecules, homoserine lactones, can promote biofilm formation in the opportunistic pathogen *Pseudomonas aeruginosa* by promoting production of biosurfactants [11,12], and by stimulating production of extracellular DNA [13] and of lectins, proteins able to promote cell adhesion to sugar moieties [14]. In addition to dedicated signal molecules, intermediates and products of different metabolic pathways can also affect biofilm formation: for instance, indole, a product of tryptophan degradation, stimulates EPS production in *Vibrio cholerae* [15]. Likewise, glucose and glycolysis intermediates can greatly impact adhesion factors' production through different regulatory mechanisms (reviewed in [16]).

In *Escherichia coli* and other enterobacteria, curli amyloid fibers greatly enhance cell aggregation and adhesion to surfaces. Genes involved in curli biosynthesis are clustered in the *csgBAC* operon, encoding curli structural components, and the *csgDEFG* operon, encoding the CsgD transcription regulator and proteins involved in curli assembly and transport [17,18]. The CsgD protein activates transcription of the *csgBAC* operon and of several genes involved in production of cell surface-associated structures and in cell adaptation to the biofilm lifestyle [19–21], including the *adrA* gene, encoding a diguanylate cyclase able to trigger cellulose production via c-di-GMP synthesis [6,22]. Thus, curli, cellulose and other cell surface-associated structures are co-produced in a CsgD-dependent fashion to constitute the biofilm extracellular

matrix. Expression of the *csg* operons takes place in response to a combination of environmental conditions: low growth temperature (<32°C), low osmolarity, and slow growth [18], and it is strongly dependent on the signal molecule c-di-GMP [7,8]. A number of regulators, including OmpR, IHF, H-NS, CpxR, Crl, and the RpoS protein, play a role in curli gene expression [18,23–25]. However, several aspects of curli regulation are still unclear: for instance, the molecular mechanisms of temperature dependence have not yet been fully elucidated, and no c-di-GMP sensor element involved in *csg* activation has been identified so far.

In this work, we show that curli and cellulose production are tightly linked to nucleotide biosynthetic pathways. In particular, transcription of the curli operons is strongly affected by pyrimidine nucleotide availability, while cellulose production is activated in the presence of exogenous uracil. Our observations suggest that production of cellulose and curli, usually co-regulated, can be unbalanced depending on the activity of different UMP biosynthetic pathway. Coupling of curli and cellulose production to UMP biosynthesis modulates formation of extracellular structures in response to physiological and environmental cues, such as starvation, nucleic acid turnover, and availability of exogenous pyrimidines.

Results

Mutations in the *carB* gene affect curli production

Amyloid fibers such as curli bind to the dye Congo red very efficiently [17]; thus, phenotype on Congo red-supplemented agar medium (CR medium, see Materials and Methods) provides a convenient method for curli detection and an easy way to screen mutants affected in curli production (Figure 1). To identify novel genes involved in curli regulation, we carried out transposon mutagenesis in the *E. coli* strain MG1655; mutants were screened for their phenotype on CR medium both at 30°C and 37°C, *i.e.*, at permissive and non-permissive temperature for curli production. Several mutants were isolated that showed altered phenotype on CR medium (data not shown): one mutant displaying a dark red phenotype at 30°C and a weak red coloration at 37°C, suggesting increased curli production (Figure 1), was further characterized. Mapping of the Tn5<R6Kγori/KAN-2> transposon indicated that the insertion site lay in the *carB* gene, encoding a subunit of carbamoyl phosphate synthetase, which catalyzes the first step in the *de novo* pyrimidine nucleotide biosynthetic pathway (Figure 2). To verify that changes in phenotype in the *carB::Tn5kan* mutant of MG1655 were indeed due to altered curli production, we transduced the mutation in a strain unable to produce curli: the MG1655*carB::Tn5kan ΔcsgA::cat* double mutant displayed a white phenotype on CR medium both at 30°C and at 37°C (Figure 1), thus indicating that the dark red phenotype of the MG1655*carB::Tn5kan* mutant is totally dependent on curli fibers.

Several pieces of evidence indicated that the *carB::Tn5kan* mutation does not result in the inactivation of carbamoyl phosphate synthase activity: the MG1655*carB::Tn5kan* mutant was not auxotrophic for pyrimidines, nor did it show any defect in growth rate on minimal medium. Finally, its phenotype on CR medium was not reversed by complementation with the wild type *carB* allele on a multicopy plasmid (data not shown). The insertion site for the *Tn5kan* transposon occurs at nucleotide 2720 of the *carB* gene, corresponding to the 907th codon, likely resulting in the production of a truncated form of the CarB protein lacking its regulatory domain involved in allosteric inhibition of protein activity by UMP [26]. Loss of the regulatory domain suggests that CarB protein activity might be increased in the MG1655*carB::Tn5-kan* mutant strain. To verify this hypothesis, we constructed a *carB* mutant in which the portion of the gene encoding the catalytic domain of the CarB protein had been deleted (MG1655*ΔcarB::cat*). As expected, this mutant was auxotrophic for pyrimidines, and showed reduced growth rate in LB1/4 medium (data not shown). Addition of uracil at 0.25 mM to LB1/4 medium (LB1/4(ura)) fully overcame MG1655*ΔcarB::cat* partial growth defect (data not shown). The MG1655*ΔcarB::cat* mutant displayed a white phenotype on CR medium, suggesting inability to produce curli (Figure 1), thus confirming the hypothesis that the *carB::Tn5kan* mutation does indeed result in enhanced carbamoyl phosphate synthetase activity.

Inactivation of UMP biosynthetic genes inhibits curli production at gene transcription level

To investigate whether the effects of *carB* inactivation could also be observed for other genes belonging to the *de novo* UMP biosynthetic pathway, we constructed knock out mutants in the *pyrB*, *pyrC* and *pyrE* genes, and tested them for their phenotypes on CR medium. As shown in Figure 3, inactivation of any UMP biosynthetic gene resulted in white phenotype on CR medium,

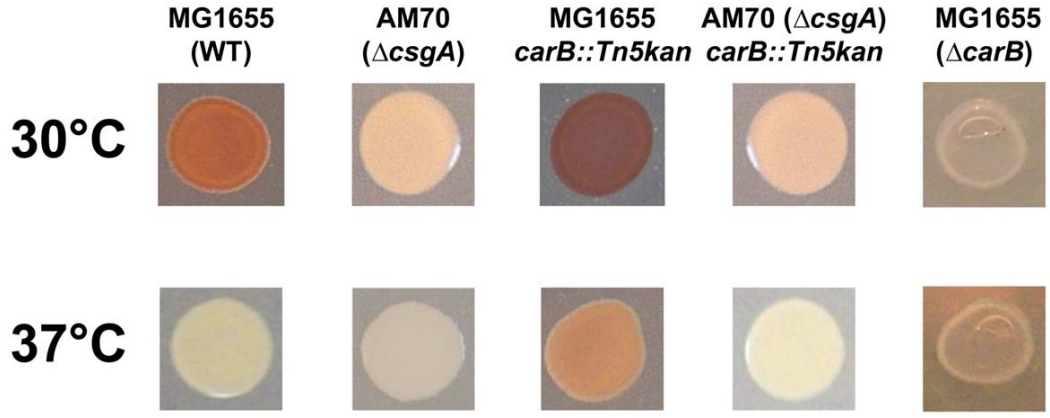

Figure 1. Determination of curli production by Congo red binding. Phenotypes on CR medium of MG1655 (wild type strain), AM70 (*csgA* deletion mutant, unable to produce curli), MG1655*carB::Tn5kan*, MG1655*carB::Tn5kan ΔcsgA::cat* and MG1655*ΔcarB::cat*. Strains were grown either at 30°C (for 24 hours) or at 37°C (for 18 hours). Plates were incubated for 48 hours at 4°C to enhance Congo red binding.
doi:10.1371/journal.pone.0031252.g001

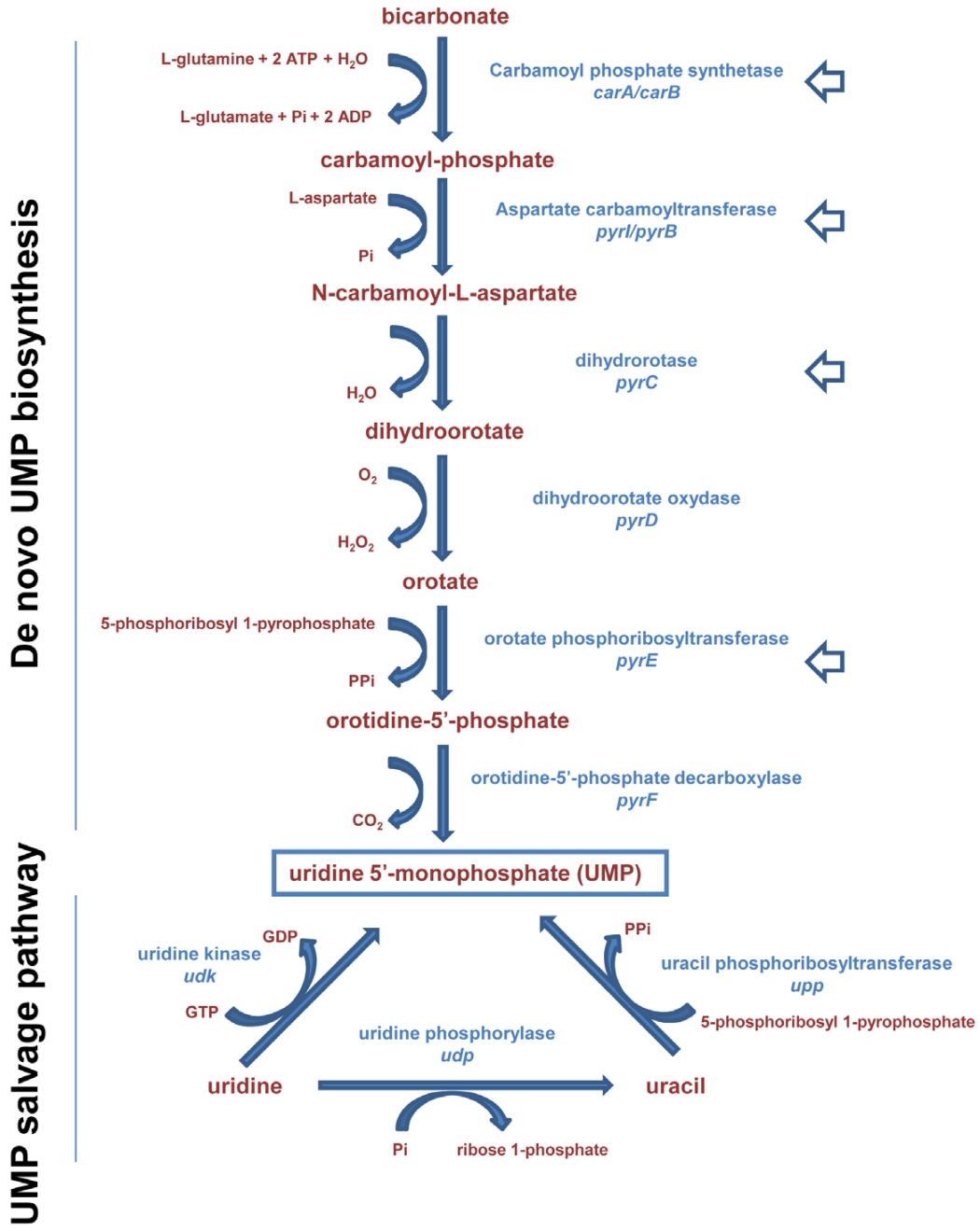

Figure 2. UMP biosynthetic pathways in *Escherichia coli*. Adapted from Ecocyc (http://ecocyc.org/).
doi:10.1371/journal.pone.0031252.g002

indicating that curli production is inhibited by pyrimidine nucleotide starvation, rather than by lack (or accumulation) of any specific intermediate in the UMP biosynthetic pathway. Consistent with this result, strains impaired in *de novo* UMP biosynthesis were deficient in surface attachment experiments (Figure S1). To elucidate the mechanism of curli inhibition by perturbation of UMP biosynthesis, we measured transcript levels of the *csgD* and *csgB* genes, representatives of the two curli biosynthetic operons, using quantitative Real Time PCR (Table 1). Transcript levels of both *csgD* and *csgB* genes were dramatically decreased in every mutant deficient in UMP biosynthesis; in contrast, they were increased by approximately 3.5-fold in the MG1655*carB::Tn5kan*, in agreement with the dark red phenotype observed in this mutant (Figure 1). Consistent with inhibition of *csgDEFG* transcription, transcript levels of the CsgD-dependent *adrA* gene were also reduced by roughly 10-fold by mutations negatively affecting *de novo* UMP biosynthesis (Table 1).

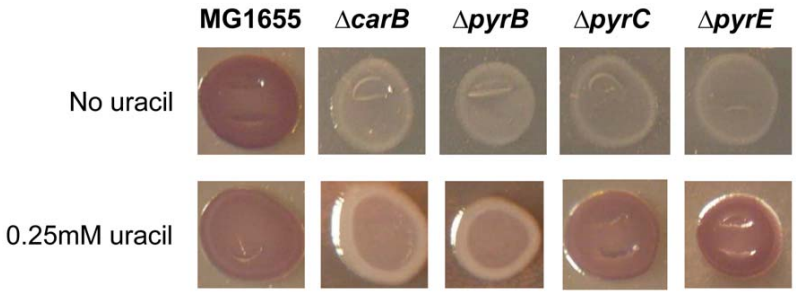

Figure 3. Congo red binding by *E. coli* strains deficient in UMP biosynthesis. The MG1655 strain and isogenic mutants deficient in UMP biosynthetic genes were spotted on either CR medium or CR(ura) medium (CR medium supplemented with 0.25 mM uracil) and grown for 24 hours at 30°C. Plates were incubated for 48 hours at 4°C to enhance Congo red binding.
doi:10.1371/journal.pone.0031252.g003

In contrast to the genes belonging to the CsgD regulon, relative amounts of 16S rRNA, used as reference gene in Real Time PCR experiments, were similar in MG1655 and in the strains carrying non-functional alleles of UMP biosynthetic genes (data not shown), as were transcript levels of the cellulose biosynthetic *bcsA* gene, which is not regulated by the CsgD protein [27,28] (Table 1). These results strongly suggest that pyrimidine starvation leads to a reduction in *csgD* and *csgB* transcript levels via a specific mechanism rather than through a general inhibition of transcription. To determine whether reduction in *csgD* transcript levels could depend on decreased mRNA stability, we performed mRNA decay experiments, which did not show any significant difference in *csgD* mRNA half-lives in MG1655*ΔcarB::cat* in comparison to MG1655 (data not shown), suggesting that knock out mutations in the *de novo* UMP biosynthetic pathway affects *csgD* expression at the transcription initiation step.

Upon addition of 0.25 mM uracil to LB1/4 medium (LB1/4(ura) medium) transcription of both *csgD* and *csgB* was re-established in mutant strains affected in *de novo* UMP biosynthesis (Table 1), thus confirming that *csgDEFG* expression is repressed by pyrimidine starvation. However, surprisingly, addition of uracil to CR medium (CR(ura) medium) failed to restore the curli-dependent red phenotype in the *carB* and *pyrB* strains (Figure 3), in apparent contradiction with the results of the gene expression experiments. In contrast, the MG1655 strain, as well as the MG1655*ΔpyrC* and MG1655*ΔpyrE* mutants, displayed a red phenotype on CR(ura) medium, which was not affected by supplementing uracil up to a final concentration of 1 mM (data not shown). Surface adhesion experiments showed that growth in LB1/4(ura) only partially restored ability to form biofilm in the MG1655*ΔcarB::cat* and MG1655*ΔpyrB::cat* strains (Figure S1). These results could suggest that, although curli operon transcription was fully resumed in the presence of additional uracil, curli subunit production might still be impaired in the MG1655*ΔcarB::cat* and MG1655*ΔpyrB::cat* strains. However, determination of curli fibers' production using the SDS-agarose electrophoresis method [29] performed on MG1655*ΔcarB::cat* showed that was fully competent for curli production when grown in LB1/4(ura) solid medium (Figure S2), in agreement with gene expression experiments (Table 1).

Effects of regulatory proteins affecting pyrimidine metabolism and of inhibition of purine biosynthesis on curli production

We investigated whether pyrimidine starvation might affect curli production and *csg* gene expression via known pyrimidine-sensing regulatory proteins. To this aim, we constructed isogenic mutants of MG1655 in which either the *cytR* or the *rutR* gene were inactivated. The CytR protein is a repressor of genes involved in pyrimidine uptake and degradation; negative regulation by CytR is relieved by high intracellular concentrations of cytidine [30]. Interestingly, in *Vibrio cholerae*, a CytR-like protein negatively controls biofilm formation by repressing EPS production [31]. DNA binding by RutR, a regulator of genes involved both in pyrimidine biosynthesis and degradation, is inhibited by uracil

Table 1. Determination of gene expression levels.

	LB1/4				LB1/4(ura)			
	csgD	*csgB*	*adrA*	*bcsA*	*csgD*	*csgB*	*adrA*	*bcsA*
MG1655	100*	100*	100*	100*	84.7	81.8	107	79.6
MG1655*carB::Tn5kan*	386	352	227	106	101	114	119	88.5
MG1655*ΔcarB::cat*	1.3	0.7	12.5	85.4	91.2	90.7	102	92.9
MG1655*ΔpyrB::cat*	0.7	0.1	n.d.	88.6	100.3	78.4	n.d.	102.5
MG1655*ΔpyrC::cat*	0.8	0.1	10.8	91.4	90.6	92.1	113	83.1
MG1655*ΔpyrE::cat*	0.5	0.2	n.d.	82.1	83.4	86.2	n.d.	86.4

Relative expression of the *csgD*, *csgB*, *adrA* and *bcsA* genes determined by Real-Time PCR on RNA extracted from overnight cultures. 16S RNA transcript was used as reference gene. *Δ*Ct values between the genes of interest and 16S RNA were set at 100 for MG1655 in LB1/4 medium, and transcript levels in other strains and/or growth conditions are expressed as relative values. Experiments were repeated at least three times, each time in duplicate; standard deviations were always lower than 5%.
doi:10.1371/journal.pone.0031252.t001

[32]. Thus, both CytR and RutR proteins regulate gene expression in response to intracellular pyrimidine concentrations. We tested the effects of the *cytR* and of the *rutR* mutations on CR phenotype, either in the presence or in the absence of exogenous uracil (Figure 4A): inactivation of the *rutR* gene did not affect CR phenotype, while, in contrast, the MG1655*ΔcytR* mutant strain displayed a white phenotype both on CR and on CR(ura) medium, indicative of reduced curli production. *csgD* transcript levels are reduced by roughly 5-fold in the *ΔcytR* mutant strain grown in LB1/4 medium, but they are restored to wild type levels by addition of 0.25 mM uracil (Figure 4B). In contrast, expression of the CytR-dependent *udp* gene, used as a control in gene expression experiments, are increased in the *cytR* mutant regardless of the presence of exogenous uracil, as expected (Figure 4B). Thus, the behavior of the *cytR* mutant with respect to curli production and *csgD* gene expression strongly resembles the MG1655*ΔcarB::cat* and MG1655*ΔpyrB::cat* strains (see Figure 3 and Table 1). These observations suggest that the CytR protein does not mediate pyrimidine-dependent regulation of the *csg* operons directly; however, lack of a functional *cytR* gene likely results in altered intracellular pyrimidine concentrations, which would in turn affect *csgDEFG* expression and curli production.

Our results show that pyrimidine starvation-dependent down-regulation of *csgDEFG* expression and of curli production is not mediated by regulatory proteins directly involved in sensing intracellular pyrimidine concentrations. Thus, we hypothesized that pyrimidine starvation might downregulate *csgDEFG* expression through a general effect on intracellular nucleotide pools. As an initial verification of this hypothesis, we tested the effects of purine starvation on curli production and *csgDEFG* expression. Inactivation of the purine biosynthetic gene *purH* resulted in white phenotype on CR medium (Figure 4A) and in a 7-fold reduction of *csgDEFG* transcript levels (Figure 4B). Similar to what observed for mutations in *de novo* pyrimidine biosynthesis, *purH* inactivation does not result in a non-specific downregulation of transcription, since transcript levels of the CsgD-independent *udp* gene were unaffected in the *purH* mutant strain (Figure 4B). As expected, addition of 0.25 mM uracil did not revert the effects of the *purH* mutation (Figure 4), indicating that uracil can only counteract the effects of mutations specifically affecting UMP concentrations.

Since curli production and *csgDEFG* expression are strongly dependent upon the signal molecule c-di-GMP [7,8], it is conceivable that changes in the nucleotide pools due to mutation in nucleotide biosynthetic genes could affect c-di-GMP production. This would be in agreement with our previous observations that sulfathiazole, a sulfonamide drug interfering with nucleotide biosynthesis, can inhibit c-di-GMP biosynthesis [33]. Determination of intracellular c-di-GMP concentrations did not show significant differences in MG1655*ΔcarB::cat* and the MG1655*carB::Tn5kan* strains in comparison to MG1655 (data not shown); however, c-di-GMP concentrations in MG1655 cells are in the nanomolar range [33], making a

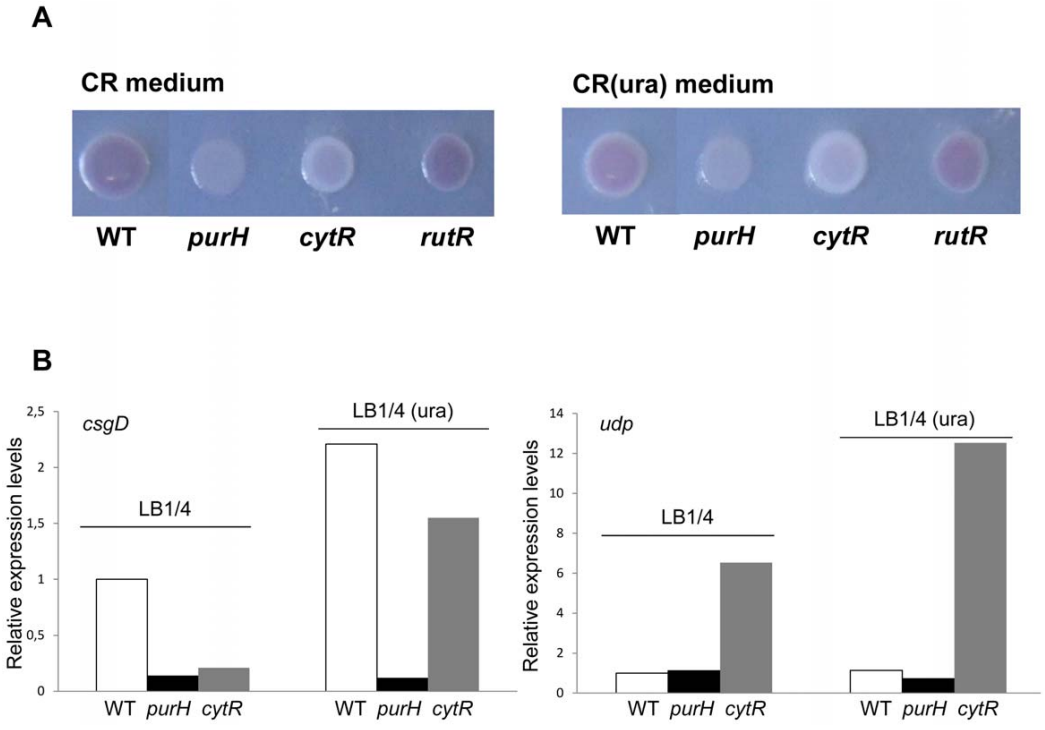

Figure 4. Congo red binding by *E. coli* strains deficient in pyrimidine sensing (*cytR* and *rutR* mutants) and purine biosynthesis (*purH* mutant). 4A. The MG1655 strain and its isogenic mutants in the *purH*, *cytR* and *rutR* genes were spotted either on CR medium (left panel) or on CR(ura) medium (right panel) and grown for 24 hours at 30°C. Plates were incubated for 48 hours at 4°C to enhance Congo red binding. Determination of transcript levels. **4B.** Relative expression of either the *csgD* gene (left panel) or the *udp* gene (right panel) was determined by Real-Time PCR on RNA extracted from overnight cultures of MG1655 and of its isogenic *purH* and *cytR* mutants. 16S RNA transcript was used as reference gene. *Δ*Ct values between the genes of interest and 16S RNA were set at 1 for MG1655 in LB1/4 medium, and transcript levels in other strains and/or growth conditions are expressed as relative values. Experiments were repeated at least three times, each time in duplicate; standard deviations were always lower than 5%.
doi:10.1371/journal.pone.0031252.g004

precise determination of c-di-GMP in cell extracts rather difficult. In addition, it must be pointed out that induction of one specific diguanylate cyclase, sufficient for activation of its corresponding target, might not result in any significant increase in the overall concentration of intracellular c-di-GMP.

Uracil triggers cellulose production

Results presented in this work (Figure 3, Table 1, Figure S1) suggest that, in the MG1655*ΔcarB::cat* and MG1655*ΔpyrB::cat* strains grown in LB1/4(ura) medium, exposure of curli fibers on the cell surface might be hindered by production of additional extracellular structures. Indeed, it is known that overproduction of cellulose and other EPS can prevent curli-mediated Congo red binding and cell adhesion [34–37]. To test the possibility that exogenous uracil might affect phenotypes on CR medium in the MG1655*ΔcarB::cat* and the MG1655*ΔpyrB::cat* strains via cellulose overproduction, we inactivated *bcsA*, the first gene of the cellulose biosynthetic operon, in these genetic backgrounds. Deletion of the *bcsA* gene restored, albeit partially, the red phenotype on CR(ura) medium (Figure 5, data not shown), suggesting that the white phenotype on CR(ura) medium might indeed depend on EPS overproduction. Likewise, it resulted in efficient surface attachment by the MG1655*ΔcarB::cat ΔbcsA::kan* double mutant (Figure S1). In contrast, deletion of the *bcsA* gene did not affect either CR phenotype or surface attachment in the MG1655 strain (data not shown), in agreement with previous observations [35]. To confirm our hypothesis further, we determined cellulose amounts in the MG1655, MG1655*ΔcarB::cat* and MG1655*ΔpyrC::tet* strains grown either in LB1/4 or in LB1/4(ura). Although growth in LB1/4(ura) enhanced cellulose production in all strains tested, this effect was much stronger in MG1655*ΔcarB::cat*, leading to production of a 3.5-fold higher amount of cellulose in comparison to MG1655 grown in the same conditions (Figure 6).

Enzymatic activity of the cellulose biosynthetic machinery is subject to regulation by the signal molecule c-di-GMP. Two distinct c-di-GMP synthetases, the AdrA and YedQ proteins, can activate cellulose production; although AdrA overexpression has been reported to affect curli production [7,35], in physiological conditions AdrA sole function is to activate cellulose production [27,37]. AdrA and YedQ act independently and belong to two distinct regulatory circuits [6,27,38]: indeed, while AdrA is encoded by a CsgD-dependent gene [22], thus presiding to co-ordinated production of curli and cellulose, YedQ expression and activity are independent of CsgD [38,39]. We tested the hypothesis that exogenous uracil might affect cellulose production via c-di-GMP synthesis by either AdrA or YedQ. Interestingly, inactivation of the *yedQ* gene, but not of *adrA*, in either the MG1655*ΔcarB::cat* (Figure 5) or the MG1655*ΔpyrB::cat* (data not shown) genetic backgrounds partially restored red phenotypes on CR medium, similar to the MG1655*ΔcarB::cat ΔbcsA* double mutant, thus suggesting that cellulose overproduction in the presence of exogenous uracil is mediated by the YedQ protein.

Discussion

In this work, we have shown that mutations in genes belonging to *de novo* nucleotide biosynthetic pathways strongly affect *csgDEFG* expression and curli production in *E. coli* (Figure 1, Figures 3–4, Table 1, Figures S1, S2). Interplay between nucleotide metabolism and biofilm appears to be conserved in different bacteria; however, specific effects and mechanism may vary substantially. Indeed, although our results are consistent with previous findings showing that active *de novo* UMP biosynthesis is necessary for biofilm formation in *P. aeruginosa* [40,41], in this bacterium inhibition of purine biosynthesis through inactivation of the *purH* gene does not affect adhesion factors' production [41], in contrast to what observed in *E. coli* (Figure 4). Likewise, pyrimidines appear to control EPS production and biofilm formation in *V. cholerae* through the dedicated regulator CytR [31], which does not appear to play a direct role in curli regulation in *E. coli* (Figure 4). Despite these differences, it seems that absence of *de novo* pyrimidine biosynthesis can act as a signal for severe nutrient starvation, which can in turn prevent biofilm formation and promote biofilm dispersal [42].

In *E. coli*, the effects of mutations in the *de novo* UMP biosynthesis on curli production can be complemented by supplementing growth medium with uracil, thus suggesting that pyrimidine nucleotide availability, regardless whether it is achieved via *de novo* UMP biosynthesis or the pyrimidine salvage pathway, allows efficient *csgDEFG* transcription and expression of the CsgD regulon (Table 1, Figure S2). Regulation of *csgDEFG* expression by intracellular nucleotide concentrations might take place by direct modulation of transcription initiation by RNA polymerase, similar to transcription control by GTP availability described for ribosomal promoters [43], or through not yet identified nucleotide-sensing regulatory proteins. Alternatively, perturbations in nucleotide pools might affect accumulation of c-di-GMP, a signal molecule necessary for *csgDEFG* expression [7,8], possibly by impairing diguanylate cyclases' enzymatic activity.

Diguanylate cyclases play a role in pyrimidine-dependent regulation of cellulose production. Cellulose production is

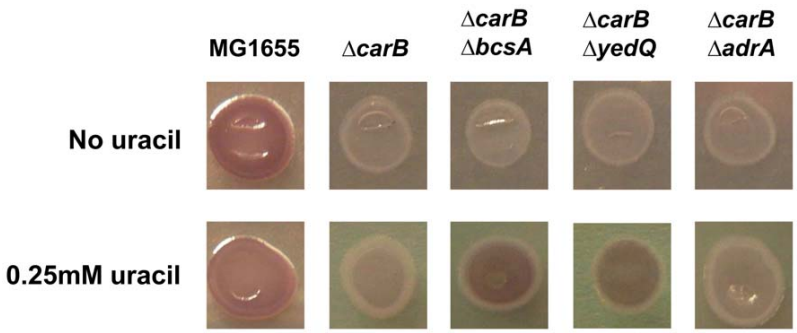

Figure 5. Effect of cellulose production on Congo red binding. Phenotypes on CR medium of MG1655, MG1655*ΔcarB::cat*, MG1655*ΔcarB::cat ΔbcsA::kan*, MG1655*ΔcarB::cat ΔyedQ::kan*, MG1655*ΔcarB::cat ΔadrA::kan*. Strains were spotted on either CR medium or CR(ura) medium (CR medium supplemented with 0.25 mM uracil) and grown for 24 hours at 30°C. Plates were incubated for 48 hours at 4°C to enhance Congo red binding.
doi:10.1371/journal.pone.0031252.g005

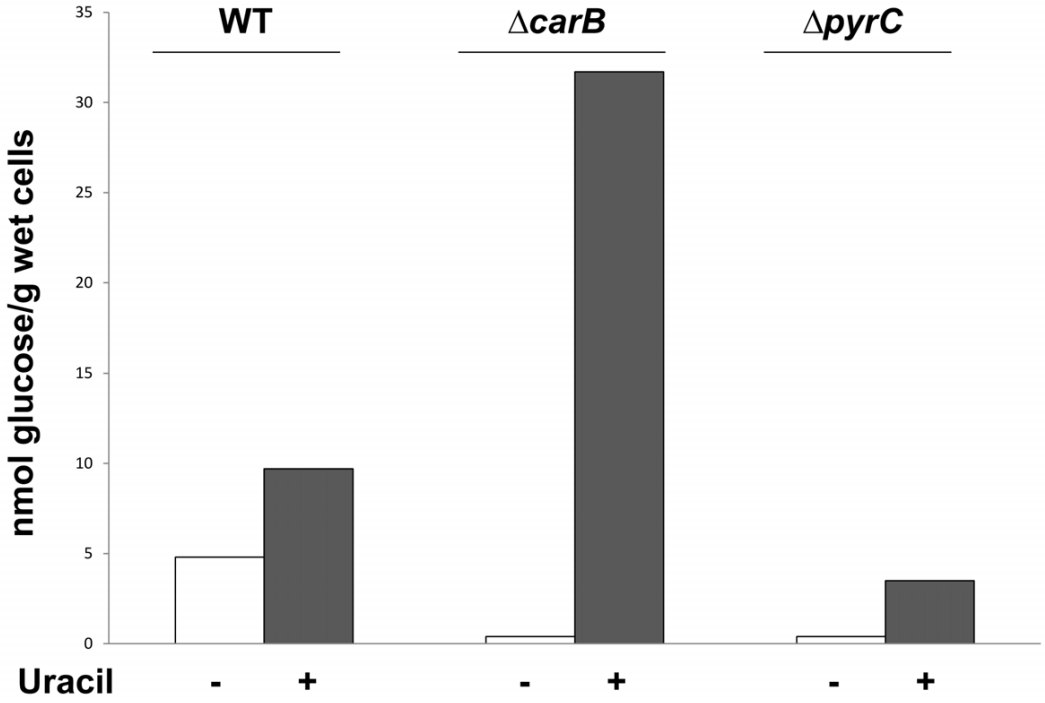

Figure 6. Determination of cellulose amounts. Strains MG1655, MG1655*ΔcarB::cat*, and MG1655*ΔpyrC::tet* were grown 48 hours at 30°C on either LB1/4 agar (no added uracil, Cellulose extraction and determination was performed as described [35]. Data shown are the average of two independent experiments giving very similar results. For strains MG1655*ΔcarB::cat* and MG1655*ΔpyrC::tet* grown on LB1/4 agar no glucose was detectable in the assays. A value of 0.5 nmol glucose, corresponding to the lowest detectable concentration in the assay, as determined by a glucose standard curve, was thus arbitrarily assigned to these strains.
doi:10.1371/journal.pone.0031252.g006

regulated by a more complex mechanism since, in addition to pyrimidine availability, it seems to respond to the relative activity of the two UMP biosynthetic pathways. Indeed, MG1655 produces twice as much cellulose when grown in the presence of exogenous uracil (Figure 6), *i.e.*, in conditions in which UMP biosynthesis is mostly carried out via the pyrimidine salvage pathway and *de novo* UMP biosynthesis is inhibited [32,44]. Induction of cellulose production by exogenous uracil is further enhanced in mutants carrying non-functional *carB* or *pyrB* alleles (Figures 3, 5–6 and data not shown): in contrast, strains carrying mutations in later steps of the *de novo* UMP biosynthetic pathway, such as MG1655*ΔpyrC::tet*, do not overproduce cellulose in response to uracil (Figure 3, Figure 6). These observations suggest that bacterial cells might sense the molecular ratio between UMP and intermediates in the *de novo* UMP biosynthesis such as carbamoyl-L-aspartate, which accumulates in the *pyrC* mutant strain, as a signal of the relative balance between the two UMP biosynthetic pathways. An unbalance towards UMP biosynthesis via the pyrimidine salvage pathway triggers cellulose production, and this effect relies on the activity of the diguanylate cyclase YedQ (Figure 5).

The interplay between nucleotide salvage pathway and cellulose production might be connected to the role of cellulose and other EPS in the response to environmental stresses such as desiccation and resistance to bacteriophages [3,35,45]. In bacterial biofilms, events leading to extensive cell lysis, such as exposure to antibiotics or attack by bacteriophages, would release cell components into the local environment: thus, a sudden increase in concentrations of exogenous nucleotides due to bacterial lysis might function as an "alarm signal" to neighboring cells, which would react by producing EPS as a defense mechanism against environmental stresses. For intracellular pathogenic enterobacteria, sensing an increase of exogenous nucleotide concentration might instead signal stress events in the host cell, such as leakage of nucleotides from the nuclear compartment. Consistent with our observations, it has been reported that allosteric inhibition of the CarB protein by exogenous uracil strongly influences production of extracellular structures and negatively affects expression of type III secretion systems in the intracellular pathogen *Shigella flexneri* [46]. In *Pseudomonas fluorescens*, a spontaneous mutation in the *carB* gene affects the proportion of capsulated and non-capsulated subpopulations via yet unknown molecular mechanisms [47]. Our results complement and expand these observations, and underline the importance of the interplay linking biofilm formation, bacterial virulence, production of extracellular structures, and nucleotide biosynthetic pathways: better understanding of these connections at the molecular level will allow us to improve our strategies in preventing (or promoting) bacterial biofilms. In this perspective, our results provide strong evidence to confirm previous findings suggesting that drugs targeting nucleotide biosynthetic pathways have a strong potential as antibiofilm agents [33,41].

Materials and Methods

Bacterial strains and growth conditions

Bacterial strains used in this work are listed in Table 2. For strain construction and manipulation, bacteria were grown in LB medium (10 g/L Tryptone, 5 g/L Yeast Extract, 5 g/L NaCl).

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

Escherichia coli Strains	Relevant genotype or characteristics	Reference or source
MG1655	K-12, F^- λ^- *rph-1*	Standard laboratory strain [52]
AM70	MG1655 *ΔcsgA::cat*	[37]
LG28	MG1655 *ΔbcsA::kan*	[35]
LG30	MG1655 *ΔadrA::kan* obtained by P1 transduction from 3934*adrA* [38]	This work
MG1655*carB::Tn5kan*	*Tn5::kan* transposon inserted at nucleotide 2720 of the *carB* gene	This work
MG1655*ΔcarB::cat*	Replacement of the nucleotides 1–550 of the *carB* gene with a chloramphenicol resistance cassette	This work
MG1655*ΔcytR::cat*	Replacement of the *cytR* gene with a chloramphenicol resistance cassette	This work
MG1655*ΔpurH::cat*	Replacement of the *purH* gene with a chloramphenicol resistance cassette	This work
MG1655*ΔpyrB::cat*	Replacement of the *pyrB* gene with a chloramphenicol resistance cassette	This work
MG1655*ΔpyrC::tet*	Replacement of the *pyrC* gene with a tetracycline resistance cassette	This work
MG1655*ΔpyrE::tet*	Replacement of the *pyrE* gene with a tetracycline resistance cassette	This work
MG1655*ΔrutR::cat*	Replacement of the *rutR* gene with a chloramphenicol resistance cassette	This work
MG1655*ΔyedQ::kan*	Replacement of the *yedQ* gene with a kanamycin cassette	This work
MG1655*carB::Tn5kan ΔcsgA::cat*	Obtained by P1 transduction from AM70 into MG1655*carB::Tn5kan*	This work
MG1655*ΔcarB::cat ΔbcsA::kan*	Obtained by P1 transduction from LG28 into MG1655*ΔcarB::cat*	This work
MG1655*ΔcarB::cat ΔadrA::kan*	Obtained by P1 transduction from LG30 into MG1655*ΔcarB::cat*	This work
MG1655*ΔcarB::cat ΔyedQ::kan*	Obtained by inactivation of the *yedQ* gene by λ red technique	This work
MG1655*ΔpyrB::cat ΔbcsA::kan*	Obtained by P1 transduction from LG28 into MG1655*ΔpyrB::cat*	This work
MG1655*ΔpyrB::cat ΔadrA::kan*	Obtained by P1 transduction from LG30 into MG1655*ΔpyrB::cat*	This work
MG1655*ΔpyrB::cat ΔyedQ::kan*	Obtained by inactivation of the *yedQ* gene by λ red technique	This work
Plasmids		
pCR2.1	Control vector allowing direct cloning of PCR products, ampicillin resistance	Invitrogen
pCR2.1-*carB*	*carB* gene cloned as PCR product into pCR2.1 vector	This work

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For adhesion assays and gene expression regulation studies, bacteria were grown in LB medium diluted 1:4 in H_2O (LB1/4). The LB1/4 medium was used since it allows efficient induction of the CsgD regulon [48] and provides sufficient pyrimidines to partially overcome the growth defect of strains mutated in *de novo* UMP biosynthetic pathway. When required, LB1/4 medium was supplemented with 0.25 mM uracil (LB1/4(ura) medium); uracil was dissolved in 50% dimethyl sulfoxide (DMSO) in water at a concentration of 10 mM. DMSO to a 1.25% final concentration was always added to control cultures.

For Congo red (CR) binding assays, overnight cultures were spotted, using a replicator, on LB1/4 agar medium to which 0.004% Congo red and 0.002% Coomassie blue were added after autoclaving (CR medium). Bacteria were grown for 20 h at 30°C; phenotypes were better detectable after 24–48 h incubation at 4°C. 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.

Genetic techniques

Transposon insertion mutagenesis was carried out using the EZ-Tn5<R6Kγori/KAN-2> transposome (Epicentre). Transposon mutagenesis and determination of transposon insertion site by rescue cloning were carried out according to the manufacturer's instructions. *E. coli* MG1655 mutant derivatives were constructed either using the λ Red technique [49] or by bacteriophage P1 transduction [50]. The list of primers used for gene inactivation and for confirmation of target gene disruption by PCR is presented in Table S1. Construction of the pCR2.1-*carB* plasmid was carried out by PCR amplification of the *carB* gene from the MG1655 genome followed by direct cloning of the PCR product into the pCR2.1vector (Invitrogen).

Gene expression studies

Determination of relative gene expression levels was performed by quantitative Real Time PCR, using bacterial cultures grown either in LB1/4 or in LB1/4(ura) at 30°C, and harvested either from overnight cultures or from exponential phase ($OD_{600\ nm} = 0.6$ for MG1655 and MG1655*carB::Tn5kan*, $OD_{600\ nm} = 0.2$ for strains carrying null mutations in UMP biosynthetic genes). Primers for Real-Time PCR are listed in Table S1. mRNA stability was measured by Real-Time PCR experiments in the presence of rifampicin as described [51]. 16S RNA was always used as reference gene.

Other methods

Detection of curli amyloid fibers was performed using the SDS-agarose electrophoresis method as described [29]. Cellulose amount was estimated on bacterial cultures grown on solid medium for 48 hours; cells were collected, resuspended in H_2O and centrifuged at 12.000×g for 10 minutes; cellulose was determined as glucose released from cellulase treatment on culture supernatants as previously described [35]. Biofilm formation was determined with

the surface attachment assay in microtiter plates [35] performed on bacterial cultures grown overnight in LB1/4 at 30°C.

Supporting Information

Figure S1 Surface adhesion on polystyrene microtiter plates. Surface adhesion experiments were performed as previously described [35]. White bars: overnight cultures grown in LB1/4 medium; grey bars: overnight cultures grown in LB1/4(ura) medium. Three independent experiments were performed and standard deviations are shown.
(TIF)

Figure S2 SDS-agarose gel. Curli production was detected using the SDS-agarose gel method [29]. The same amount of total protein was loaded in each sample. Insoluble material, mostly constituted by curli amyloids, cannot migrate into the agarose gel and is stained by Coomassie blue. Cultures were grown on solid medium (LB1/4 agar or LB1/4(ura) agar) for 24 hours at 30°C.
(TIF)

Table S1 Primers used in this work.
(DOC)

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Author Contributions

Conceived and designed the experiments: MG ER PL. Performed the experiments: MG ER PL. Analyzed the data: MG ER PL. Contributed reagents/materials/analysis tools: PL. Wrote the paper: PL.

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SUPPLEMENTARY TABLES

Table S1. Primers used in this work.

Primers	Sequence	Utilization
carB_for	5'-CAGTACCGTAAAACCGCT-3'	*carB* cloning and mutant verification
carB_rev	5'-ATATCTGCCATGACACGC-3'	*carB* cloning
carB_cat_for	5'-AGTACCGTAAAACCGCTAAGTAATCAGGAGTAAAAGAGCCTACCTGTGACGGAAGATCAC-3'	*carB* inactivation
carB_cat_rev	5'-AGCCCACGTCAGCGGCAACCGCCAGCGCTTCTTCCATCGTGGGCACCAATAACTGCCTTA-3'	*carB* inactivation
pyrB_cat_for	5'-TCGAGGGGCTTTTTTTTGCCCAGGCGTCAGGAGATAAAAGTACCTGTGACGGAAGATCAC-3'	*pyrB* inactivation
pyrB_cat_rev	5'-TCATCGAGCATATCCAGAATGTATTGCGGCATTGCCAGCGGGGCACCAATAACTGCCTTA-3'	*pyrB* inactivation
pyrC_tet_for	5'-GTCCGGCAAAAACATCCCTTCAGCCGGAGCATAGAGATTACTAGACATCATTAATTCCTA-3'	*pyrC* inactivation
pyrC_tet_rev	5'-CAGGTAAAATAACCTAATGACAACAGGAAGCTACGATTTGAAGCTAAATCTTCTTTATCG-3'	*pyrC* inactivation
pyrE_tet_for	5'-TTTTTTTTGTCTGTAGAAAAGTAAGATGAGGAGCGAAGGCCTAGACATCATTAATTCCTA-3'	*pyrE* inactivation
pyrE_tet_rev	5'-TAATATGACGCCGGATGACTTTTCATCCGGCGAGTTTCTGAAGCTAAATCTTCTTTATCG-3'	*pyrE* inactivation
yedQ_FRT_for	5'-GCCAGAATCATAAAAAAGCAGGTTGGGAGTCGTCAGGGTGGTGTAGGCTGGAGCTGCTTC-3'	*yedQ* inactivation
yedQ_FRT_rev	5'-GGCTGGACCATTTTTTCTCCGCCCGTTAAGCGTTATCGCTTTCCGGGGATCCGTCGACCT-3'	*yedQ* inactivation
purH_FRT_for	5'-AACGCTCTCTGTAATAGTCAAATCCAGGGGATTTACCATGGTGTAGGCTGGAGCTGCTTC-3'	*purH* inactivation
purH_FRT_rev	5'-ACTAATACTTTCATCTATTGCTCCATTAATGGCGGAAGTGTTCCGGGGATCCGTCGACCT-3'	*purH* inactivation
cytR_FRT_for	5'-GATGTAGTACGCCTGACGTGCCAGGCGAGGAGTGAGTGTGGTGTAGGCTGGAGCTGCTTC-3'	*cytR* inactivation
cytR_FRT_rev	5'-	*cytR* inactivation

	GTCACGGCAGTCTTAAAGGTTTACTTTAAGGTAA CGCGCGTTCCGGGGATCCGTCGACCT-3’	
rutR_FRT_for	5’-TGCTATCCTGTTGCCAATCTACAAGAGGGGAGAG CGCATGGTGTAGGCTGGAGCTGCTTC-3’	*rutR* inactivation
rutR_FRT_rev	5’-GATGTTACAACCTCCTCCGGCATCTTTAACGTGGT CGAATTTCCGGGGATCCGTCGACCT-3’	*rutR* inactivation
pyrB_for	5’-CTTCCCGTTGATCACCCATT-3’	Mutant verification
pyrC_for	5’-ATTTTCGTGCAAAGGAAAA-3’	Mutant verification
pyrE_for	5’-ATTTTTAAGGCGACTGAT-3’	Mutant verification
yedQ_for	5’-CGCTGTTTTGCGGTACGCTA-3’	Mutant verification
purH_for	5’-GCCCACGGTAACCACAGTCA-3’	Mutant verification
cytR_for	5’-GAGGGTTAAACCGCTCACGA-3’	Mutant verification
rutR_for	5’-GCGATGAGAGTGCAGAAGGT-3’	Mutant verification
csgA_for	5’-ACAGTCGCAAATGGCTATTC-3’	Mutant verification
bcsA_for	5’-CTAAGCAACCAGTAGGTGAATATC-3’	Mutant verification
adrA_for	5’-GCTCCGTCTCTATAATTTGGG-3’	Mutant verification
cat_rev	5’-GGGCACCAATAACTGCCTTA-3’	Mutant verification
tet_rev	5’-TGCAGGTAAAGCGATCCCACCAC-3’	Mutant verification
P2_rev	5’-TTCCGGGGATCCGTCGACCT-3’	Mutant verification
kan-2_rev	5’-ACTCTGGCGCATCGGGCTTC-3’	Transposon insertion and mutant verification
R6Kγori_for	5'-CCTCTTTCTCCGCACCCGAC-3'	Transposon insertion verification
16S_for	5’-TGTCGTCAGCTCGTGTCGTGA-3’	qRT-PCR
16S_rev	5’-ATCCCCACCTTCCTCCGGT-3’	qRT-PCR
csgD_RT_for	5’-CCCGTACCGCGACATTG-3’	qRT-PCR
csgD_RT_rev	5’-ACGTTCTTGATCCTCCATGGA-3’	qRT-PCR
csgB_RT_for	5’-CATAATTGGTCAAGCTGGGACTAA-3’	qRT-PCR
csgB_RT_rev	5’-GCAACAACCGCCAAAAGTTT-3’	qRT-PCR
adrA_RT_for	5’-GGCTGGGTCAGCTACCAG-3’	qRT-PCR
adrA_RT_rev	5’-CGTCGGTTATACACGCCCG-3’	qRT-PCR
bcsA_RT_for	5’-GACGCTGGTGGCGCTG-3’	qRT-PCR
bcsA_RT_rev	5’-GGGCCGCGAGATCACC-3’	qRT-PCR
udp_RT_for	5’-CGATTTACAAGGGGCTACGC-3’	qRT-PCR
udp_RT_rev	5’-GTGAATTCGCGGTGAGATGC-3’	qRT-PCR

SUPPLEMENTARY FIGURES

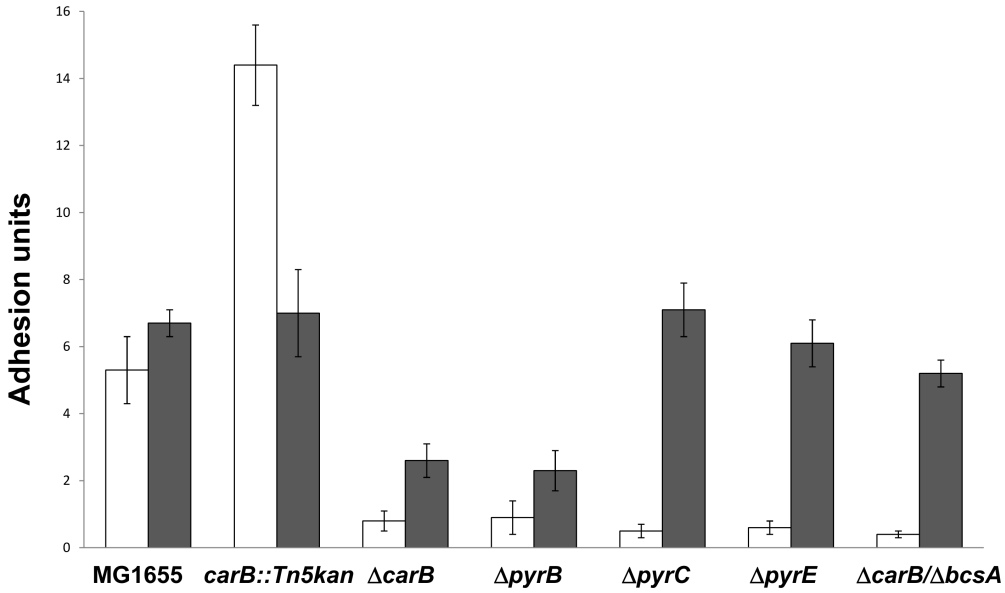

Figure S1

Figure S1. Surface adhesion on polystyrene microtiter plates. Surface adhesion experiments were performed as previously described [35]. White bars: overnight cultures grown in LB1/4 medium; grey bars: overnight cultures grown in LB1/4(ura) medium. Three independent experiments were performed and standard deviations are shown.

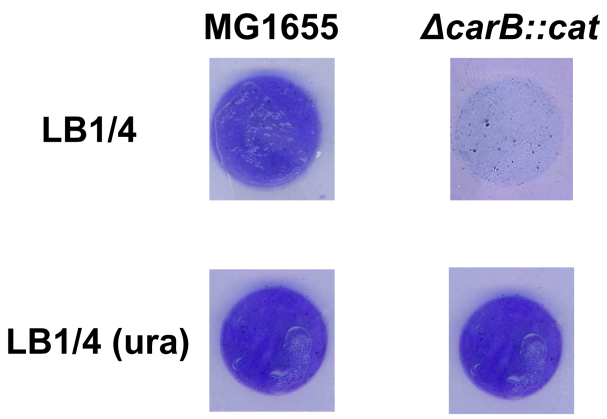

Figure S2

Figure S2. SDS-agarose gel. Curli production was detected using the SDS-agarose gel method [29]. The same amount of total protein was loaded in each sample. Insoluble material, mostly constituted by curli amyloids, cannot migrate into the agarose gel and is stained by Coomassie blue. Cultures were grown on solid medium (LB1/4 agar or LB1/4(ura) agar) for 24 hours at 30°C.

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The immunosuppressive drug azathioprine inhibits biosynthesis of the bacterial signal molecule cyclic-di-GMP by interfering with intracellular nucleotide pool availability

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Abstract In Gram-negative bacteria, production of the signal molecule c-di-GMP by diguanylate cyclases (DGCs) is a key trigger for biofilm formation, which, in turn, is often required for the development of chronic bacterial infections. Thus, DGCs represent interesting targets for new chemotherapeutic drugs with anti-biofilm activity. We searched for inhibitors of the WspR protein, a *Pseudomonas aeruginosa* DGC involved in biofilm formation and production of virulence factors, using a set of microbiological assays developed in an *Escherichia coli* strain expressing the *wspR* gene. We found that azathioprine, an immunosuppressive drug used in the treatment of Crohn's disease, was able to inhibit WspR-dependent c-di-GMP biosynthesis in bacterial cells. However, in vitro enzymatic assays ruled out direct inhibition of WspR DGC activity either by azathioprine or by its metabolic derivative 2-amino-6-mercapto-purine riboside. Azathioprine is an inhibitor of 5-aminoimidazole-4-carboxamide ribotide (AICAR) transformylase, an enzyme involved in purine biosynthesis, which suggests that inhibition of c-di-GMP biosynthesis by azathioprine may be due to perturbation of intracellular nucleotide pools. Consistent with this hypothesis, WspR activity is abolished in an *E. coli purH* mutant strain, unable to produce AICAR transformylase. Despite its effect on WspR, azathioprine failed to prevent biofilm formation by *P. aeruginosa*; however, it affected production of extracellular structures in *E. coli* clinical isolates, suggesting efficient inhibition of c-di-GMP biosynthesis in this bacterium. Our results indicate that azathioprine can prevent biofilm formation in *E. coli* through inhibition of c-di-GMP biosynthesis and suggest that such inhibition might contribute to its anti-inflammatory activity in Crohn's disease.

Keywords c-di-GMP · Diguanylate cyclase · Biofilm formation · Antimetabolite drugs · Crohn's disease · Azathioprine

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Introduction

Growth as a biofilm can reduce bacterial sensitivity to antibiotics and induce tolerance to the immune response, thus contributing to pathogenesis and to bacterial persistence in the host (Costerton et al. 1995; Romling and Balsalobre 2012). Although biofilm inhibition does not usually impair bacterial growth under laboratory conditions, treatment with biofilm-inhibiting compounds can improve growth inhibition and bacterial killing by antibiotics, thus making biofilm determinants an interesting target for specific inhibitors, to be used in combination with conventional antimicrobial agents (Jabra-Rizk et al. 2006; Rivardo et al. 2009).

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Transition of bacterial cells from planktonic to biofilm growth mode is regulated by environmental and/or physiological signals, relayed to the bacterial cell by signal molecules or "second messengers." The second messenger bis-(3′,5′)-cyclic diguanylic acid, better known as cyclic-di-GMP (c-di-GMP), plays a pivotal role in several processes linked to biofilm formation and maintenance, such as production of extracellular polysaccharides and adhesion factors (Kader et al. 2006; Weber et al. 2006; Ueda and Wood 2009), and in modulation of virulence factors (Kulasakara et al. 2006; Hammer and Bassler 2009; Fazli et al. 2011). c-di-GMP acts as a ligand and/or an allosteric effector for various protein classes, namely: transcription factors, such as FleQ in *Pseudomonas aeruginosa* (Hickman and Harwood 2008; Baraquet et al. 2012) and CpsQ in *Vibrio parahaemolyticus* (Ferreira et al. 2012), components of the biosynthetic machinery for the extracellular polysaccharides cellulose and poly-*N*-acetylglucosamine (Rijenkov et al. 2006; Steiner et al. 2012; Whitney and Howell 2012), and flagellar motor subunits (Boehm et al. 2010; Paul et al. 2010). In addition, c-di-GMP can affect gene expression regulation by direct binding to mRNA sequences, thus acting as a riboswitch (Sudarsan et al. 2008).

Intracellular c-di-GMP levels are determined by the opposite activities of diguanylate cyclases (DGCs) and c-di-GMP-phosphodiesterases (reviewed in Tamayo et al. 2007). Genes encoding proteins involved in c-di-GMP biosynthesis and turnover are conserved in all *Eubacteria* while not being present in most *Eukarya*, making DGC proteins an attractive target for specific biofilm inhibitors; they are present in remarkably high numbers in Gram-negative bacteria (Galperin 2004). Consistent with this observation, c-di-GMP was shown to be dispensable for biofilm formation in several Gram positive bacteria, such as *Staphylococcus aureus* (Holland et al. 2008; Shang et al. 2009). However, recent observations suggest a pivotal role for c-di-GMP in modulation of cell aggregation in the Gram positive pathogen *Clostridium difficile* (Purcell et al. 2012). Interestingly, c-di-GMP has been shown to counteract *S. aureus* virulence in a mastitis infection mouse model (Brouillette et al. 2005), likely due to its strong immunostimulatory activity (Karaolis et al. 2007; Gray et al. 2012).

Due to the pivotal role of c-di-GMP in biofilm formation and in production of virulence factors, DGC proteins have been targeted for development of specific inhibitors both via chemical design of substrate analogues (Spangler et al. 2011) or via high-throughput screenings (Sambanthamoorthy et al. 2012). In a previous study (Antoniani et al. 2010), we described a set of microbiological assays suitable for high-throughput screening of DGC inhibitors. This approach led to the identification of sulfathiazole, a sulfonamide antibiotic, as an inhibitor of DGC activity in vivo. In this report, we report the selection of inhibitors of WspR, a *P. aeruginosa* DGC involved in production of exopolysaccharides and in regulation of virulence (Moscoso et al. 2011). We show that azathioprine, an anti-inflammatory drug used in treatment of several autoimmune conditions, including inflammatory bowel diseases, can inhibit c-di-GMP biosynthesis by WspR in vivo. WspR inhibition appears to be indirect, and is probably due to alteration of intracellular nucleotide pools by azathioprine. We suggest that the anti-inflammatory effect of azathioprine in patients with inflammatory bowel disease might be mediated, at least in part, by inhibition of c-di-GMP biosynthesis in the bacterial cell, with consequent loss of c-di-GMP-dependent stimulation of the immune response.

Materials and methods

Bacterial strains, plasmids, and growth conditions Bacterial strains used in this work are listed in Table 1. For detection of extracellular structures, bacteria were grown on either Congo red (CR)- or Calcofluor (CF)-supplemented agar media. Both media are composed of 1 % Casamino acids, 0.15 % yeast extract, 0.005 % $MgSO_4$, 0.0005 % $MnCl_2$, and 1.5 % agar to which either 0.004 % CR and 0.002 % Coomassie blue (for CR medium) or 0.005 % CF (for CF medium) were added after autoclaving. CR and CF allow visualization, respectively, of curli fibers and cellulose, i.e., the two main extracellular structures produced by *Escherichia coli* MG1655 (Gualdi et al. 2008). For biofilm formation assays in liquid media, when not otherwise stated, bacteria were grown at 30 °C, in 1 % Casamino acids, 0.15 % yeast extract, 0.005 % $MgSO_4$, and 0.0005 % $MnCl_2$. Although CR is not present, this medium was called "liquid Congo red" medium (CRL), due to the fact that its nutrient composition is identical to the CR medium utilized for CR binding assays. For growth in minimal medium, M9/Glu (82 mM Na_2HPO_4, 24 mM KH_2PO_4, 85 mM NaCl, 19 mM NH_4Cl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.1 μg/ml thiamine, and 0.2 % glucose) was used.

Biofilm formation was assessed using the semi-quantitative crystal violet assay (CV) as previously described (Antoniani et al. 2010); relative adhesion units are determined as the $OD_{600\ nm}$ of CV-stained biofilm cells normalized to the $OD_{600\ nm}$ of the planktonic cells from the corresponding liquid cultures (Dorel et al. 1999). When needed, antibiotics were used at the following concentrations: ampicillin, 100 μg/ml and chloramphenicol, 35 μg/ml. Plasmids and primers used in this work are listed in Table 1. For overproduction of the WspR protein the corresponding gene was amplified by PCR from the *P. aeruginosa* PAO1 chromosome and the resulting product was cloned into the pTOPO vector.

Screening for WspR inhibitors The screening for WspR inhibitors was carried out as previously described for the *E. coli* DGC AdrA (Antoniani et al. 2010). Briefly, a primary

Table 1 Bacterial strains, plasmids, and primers used in this study

Bacterial strains	Relevant genotype or characteristics	Reference
Escherichia coli MG1655	Standard reference strain F^-, λ^- *rph-1*	Blattner et al. (1997)
MG1655Δ*purH*::*cat*	Replacement of the *purH* gene with a chloramphenicol resistance cassette in a MG1655 background	Garavaglia et al. (2012)
PHL856	PHL565 *csgA*::*uidA-kan*	Gualdi et al. (2008)
E. coli PHL565W	Spontaneous mutation in MG1655, deficient in curli production	This work
PHL565WΔ*purH*::*cat*	Replacement of the *purH* gene with a chloramphenicol resistance cassette in a PHL565W background	This work
E. coli 16	Clinical isolate	This work
E. coli 74	Clinical isolate	This work
Pseudomonas aeruginosa PAO1	Standard reference strain	Stover et al. (2000)
Plasmids		
pTOPO	Control vector allowing direct cloning of PCRproducts, ampicillin and kanamycin resistance	Invitrogen
pTOPOWspR	*wspR* gene from *P. aeruginosa* cloned as PCR product into pTOPO vector	This work
pTOPOYdaM	*ydaM* gene from *E. coli* cloned as PCR product into pTOPO vector	Antoniani et al. (2010)
Primers	Sequence	Utilization
wspR_fwr	5′-GGTCCCGGAGAGAAAC-3′	Amplification of the *wspR* gene
wspR_rev	5′-GCCGGCCTCTATTTAATGC-3′	Amplification of the *wspR* gene
purH_FRT_for	5′-AACGCTCTCTGTAATAGTCAAATC CAGGGGATTTACCATG**GTGTAGGCTGGAGCTGCTTC**-3′	*purH* mutant construction
purH_FRT_rev	5′-ACTAATACTTTCATCTATTGCTCCA TTAATGGCGGAAGTG**TTCCGGGGATCCGTCGACCT**-3′	*purH* mutant construction
purH_for	5′-GCCCACGGTAACCACAGTCA-3′	*purH* mutant verification
P2_rev	5′-TTCCGGGGATCCGTCGACCT-3′	*purH* mutant verification

Entries set in bold are the sequences annealing to the chloramphenicol acetyltransferase cassette (cat)

qualitative assay based on CR binding to curli was performed on solid medium in 96-well microtiter plates: 200 μl of CR medium were distributed prior to solidification to the wells, containing different chemicals; 5 μl of an overnight culture were layered on top of the solidified CR medium. The 96-well microtiter plate was incubated for 24 h at 30 °C, a temperature facilitating curli and cellulose production in *E. coli* laboratory strains (Gualdi et al. 2008; Zogaj et al. 2001; Robbe-Saule et al. 2006); staining was better detected after 24–48 h of incubation at 4 °C. Compounds showing some level of activity (i.e., partial or total colony discoloration) in the miniaturized CR assay were further tested in the semi-quantitative CV, to assess inhibition of biofilm formation, and on solid CF medium, to verify inhibition of cellulose production. A chemical library from Prestwick Chemicals (http://www.prestwickchemical.fr/index.php?pa=26) was used for the screening. Each chemical is provided at a 2-mg/ml solution in dimethylsulfoxide (DMSO) and was tested in the CR assay at 2–10–50 μg/ml.

Determination of intracellular c-di-GMP concentration Overnight cultures were collected by centrifugation and the supernatant carefully removed. For cultures treated with azathioprine, the chemical was dissolved at 10 mg/ml in 50 % DMSO; final DMSO concentration was kept at 1 % in all samples, including the untreated controls. Bacterial cells were resuspended in 0.4 M $HClO_4$ at a ratio of 45 mg cells/0.35 ml and broken by sonication; cell debris were removed by centrifugation (10,000×*g*, 10 min, 4 °C).

Supernatants were neutralized with 0.16 M K_2CO_3, kept on ice for 10 min and centrifuged at 12,000×*g* for 3 min. Supernatants were filtered and injected into a HPLC system equipped with a diode-array detector. HPLC separation was essentially performed as described in (Stocchi et al. 1985). A 12.5-cm Supelcosil LC-18-DB, 3 μm particle size, reversed-phase column was used, and the temperature was fixed at 18 °C. Elution conditions were 9 min at 100 % buffer A (100 mM potassium phosphate buffer, pH 6.0), followed by step elution to 12, 45, and 100 % buffer B (buffer A containing 20 % methanol), at a flow rate of 1.3 ml/min. Purity index of c-di-GMP peak is 0.96. Its identity as genuine c-di-GMP was determined by coelution and identical UV absorption spectra with a c-di-GMP standard (purchased from Biolog, Bremen, Germany). c-di-GMP concentration was calculated based on an extinction coefficient (ε) of 23700 at 254 nm (Hayakawa et al. 2003).

DGC purification and in vitro activity assay Purification of PleD, a DGC from *Caulobacter crescentus*, was performed as described (Paul et al. 2004). Purification of histidine-tagged WspR and removal of WspR-bound c-di-GMP by

treatment with the c-di-GMP phosphodiesterase RocR was carried out essentially as previously described (De et al. 2008). DGC activity of PleD was assayed as described previously (Paul et al. 2007). The standard reaction mixture containing 3 μM purified PleD in 20 mM Tris–HCl, pH 8.0, 100 mM NaCl, 10 mM $MgCl_2$ was incubated 30 min at room temperature with 1 mM $BeCl_2$, and 10 mM NaF (pre-activation step); as previously reported, incubation of the protein with BeF_3, a molecular mimic of phosphoryl group, promotes protein dimerization and catalytic activity (Paul et al. 2007). DGC assays for WspR were carried out essentially according to (De et al. 2008), with minor modifications; the reaction was carried out using 9 μM WspR in 25 mM Tris at pH 7.5, 100 mM NaCl, and 2 mM $MgCl_2$.

DGC activity was started by adding 100 or 500 μM GTP (25 °C), for PleD and WspR, respectively. If required, the reaction was carried out in the presence of 5 % DMSO. To calculate the initial velocity of product formation, aliquots (300 μl) were withdrawn at regular time intervals, bolied for 10 min and filtered. The c-di-GMP content was detected by reverse-phase HPLC on Prevail C8 150×4.6 mm column equilibrated with 100 mM sodium phosphate at pH 5.8, 2 % methanol. As a reference, a standard curve of synthetic c-di-GMP (Biolog) at different concentrations was also determined. Inhibition assays were carried out in the presence of 500 μM inhibitor and the reaction was stopped either after 1 h (for PleD) or 30 min (for WspR) after substrate addition.

Other methods. The *E. coli* PHL565W derivative deleted in the *purH* gene (PHL565WΔ*purH*::*cat*) was constructed using the λ Red technique (Datsenko and Wanner 2000). Target gene disruption was confirmed by PCR using primers listed in Table 1. β-glucuronidase-specific activity was measured by hydrolysis of *p*-nitrophenyl-β-D-glucuronide into *p*-nitrophenol at 405 nm (Bardonnet and Blanco 1992). Antimicrobial activity was determined as the minimal inhibitory concentration, using the broth microdilution method according to the guidelines of the Clinical and Laboratory Standard Institute (2006). Bacterial growth was assessed by spectrophotometric determination at a wavelength of 600 nm.

Results

Identification of azathioprine as inhibitor of WspR-mediated curli production. Overexpression of DGCs in *E. coli* results in increased production of the extracellular adhesion factors curli fibers and cellulose (Zogaj et al. 2001; Simm et al. 2005; Tagliabue et al. 2010). Curli production can easily be detected on agar medium supplemented with CR, a dye that binds amyloid fibers (CR medium; Zogaj et al. 2001; Antoniani et al. 2010). As part of a project aiming to identify novel inhibitors of biofilm formation by *P. aeruginosa* for the treatment of bacterial infections in cystic fibrosis patients, we set up a screening strategy targeting WspR, a *P. aeruginosa* DGC involved in production of exopolysaccharides and in regulation of virulence (Moscoso et al. 2011). The WspR protein was expressed in *E. coli*, using curli production as a reporter of WspR DGC activity. Low-level overexpression of the WspR protein was achieved by transforming *E. coli* MG1655 with the pTOPO plasmid carrying the *wspR* gene (pTOPOWspR) under the control of the P*lac* promoter, without IPTG induction. A band of ca. 38 kDa was clearly detectable in the soluble fraction of bacterial cell extract (Fig. S1 in the Electronic supplementary material (ESM)). The band was excised, and the WspR protein was identified as its major component by matrix-associated laser desorption ionization–time of flight analysis after in-gel trypsin digestion (Table S1 in the ESM). However, WspR overexpression did not result in a detectable phenotype in the MG1655 strain, which, being a curli-producing strain, displays a red phenotype on CR medium even in the absence of DGC overexpression. For this reason, the pTOPOWspR plasmid was used to transform PHL565W, a spontaneous mutant of MG1655 isolated in our laboratory: PHL565W is unable to produce curli, due to a yet uncharacterized mutation that affects transcription of the curli-encoding *csg* operons, and therefore displays white phenotype on CR medium (Fig. 1a). Production of WspR in the PHL565W restores the red phenotype on CR medium in a curli-dependent fashion: indeed, inactivation of the *csgA* gene, encoding the curli major subunit, in the PHL565W background, results in a white phenotype on CR medium even in the presence of the pTOPOWspR plasmid (Fig. 1a). Thus, the PHL565W/pTOPOWspR strain was deemed a suitable biosensor strain for a primary screening assay aimed to the selection of inhibitors of WspR DGC activity.

The Prestwick Chemical library (http://www.prestwickchemical.fr/index.php?pa=26) was used for the screening of WspR inhibitors. This library contains 1,120 chemical compounds with known biological activities, already tested for bioavailability and safety in humans, and is based on the Selective Optimization of Side Activities criteria for the identification of novel biological activities by known drugs (Wermuth 2006). Screening of the Prestwick Chemical Library on CR medium led to the identification of two active compounds: sulfathiazole and azathioprine (Fig. 1b). Their activity was confirmed both in CF binding assays, indicative of cellulose production, and in biofilm inhibition assays (Fig. 1b), thus strongly suggesting that both compounds inhibit WspR-mediated curli and cellulose production. Sulfathiazole is a sulfonamide antibiotic, which targets folic acid production and interferes with nucleotide and amino acid biosynthetic pathways; we previously demonstrated inhibition of DGC activity by sulfathiazole, using a similar screening strategy targeting the DGCs AdrA and YdaM from *E. coli* (Antoniani et al. 2010). In contrast,

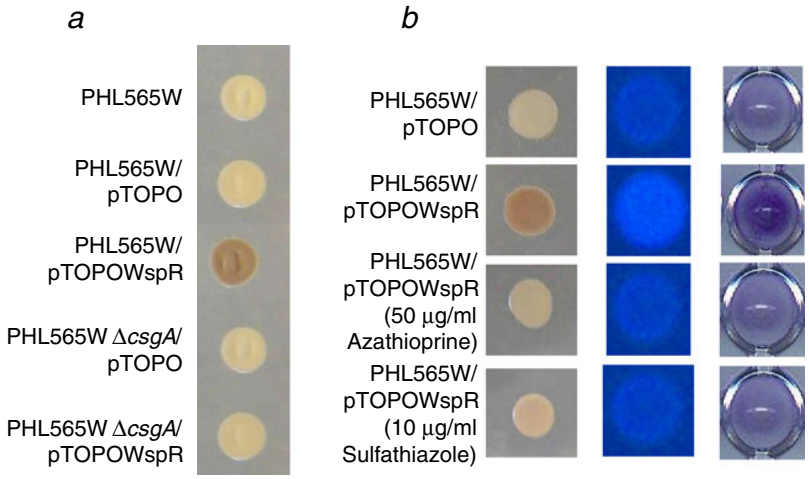

Fig. 1 **a** Effects of the expression of the WspR protein on CR binding in the PHL565W strain of *E. coli* and on a PHL565WΔ*csgA* mutant derivative unable to produce curli. **b** Inhibition of WspR-mediated production of extracellular structures and biofilm formation by azathioprine and by sulfathiazole, as measured with CR and CF binding assays and with the CV. Semi-quantitative evaluation of biofilm in CV assays (calculated as in "Materials and methods" from two independent experiments, with each sample repeated in duplicate) gave adhesion values of 0.57 for PHL565W/pTOPO, 22 for PHL565W/pTOPOWspR, 1.37 for PHL565W/pTOPOWspR in the presence of 10 μg/ml sulfathiazole, and 1.24 for PHL565W/pTOPOWspR in the presence of 50 μg/ml azathioprine

azathioprine had not been found active in our previous screening, possibly due to the higher concentrations needed for inhibition of curli production in comparison to sulfathiazole (50 vs. 10 μg/ml, Fig. 1b). Azathioprine is a purine analogue, considered to be a pro-drug for the active molecule 6-mercaptopurine and its nucleotide derivatives (Lennard 1992); azathioprine is mostly used as an immunosuppressive and anti-inflammatory drug, and is widely employed in the treatment of Crohn's disease and ulcerative colitis (Dignass et al. 2010).

Azathioprine inhibits c-di-GMP biosynthesis in whole cell assays. CR binding and biofilm formation assays (Fig. 1b) showed inhibition of WspR-dependent curli and cellulose production by azathioprine in the PHL565W/pTOPOWspR strain. Thus, we performed whole cell assays to investigate whether azathioprine impairs DGC activity by WspR. DGCs induce curli production by transcription activation of the curli-related operons *csgDEFG* and *csgBAC*, whose transcription is responsive to intracellular c-di-GMP concentrations (Kader et al. 2006; Weber et al. 2006; Sommerfeldt et al. 2009). Thus, we measured transcription levels from the P*csgBAC* promoter using a reporter strain (PHL856; Gualdi et al. 2008) that carries a *csgA*::*uidA* chromosomal transcriptional fusion. The *uidA* gene encodes β-glucuronidase, whose enzymatic activity can easily be monitored with a colorimetric assay (Bardonnet and Blanco 1992). β-glucuronidase experiments performed on overnight cultures grown in CRL medium at 30 °C showed that WspR production activates *csgBAC* transcription by roughly 6-fold (615 vs. 107 β-glucuronidase units), similar to *csgBAC* activation observed upon overexpression of the *E. coli* DGCs AdrA and YdaM (Antoniani et al. 2010). Azathioprine inhibited *csgBAC* transcription in the PHL856 strain transformed with pTOPOWspR at concentrations similar to those necessary to fully impair biofilm formation: indeed, 50 % inhibition of WspR-dependent *csgBAC* transcription was observed at 25 μg/ml (90 μM) azathioprine (Fig. 2a). In contrast, in the conditions tested, no antimicrobial activity was detectable for azathioprine up to 128 μg/ml (460 μM; Table S3 in the ESM), indicating that inhibition of *csgBAC* transcription was not due to bacterial growth inhibition.

To verify that the effects of azathioprine on curli production, biofilm formation, and *csgBAC* gene expression correlated with inhibition of c-di-GMP biosynthesis, we measured c-di-GMP intracellular concentrations by HPLC both in the absence and in the presence of different azathioprine concentrations. WspR DGC activity in the PHL565W/pTOPOWspR strain increased intracellular c-di-GMP concentrations by 11-fold compared with PHL565W/pTOPO (30.7 vs. 2.8 nmol/g dry weight). Exposure to azathioprine resulted in 50 % reduction in c-di-GMP intracellular levels at 25 μg/ml (90 μM), i.e., at the same concentration needed for 50 % inhibition of *csgA* gene expression (Fig. 2a). In contrast, exposure to azathioprine only led to a moderate decrease of intracellular pools of other nucleotides. Indeed, although the HPLC separation protocol used for c-di-GMP determination does not allow precise quantification of GTP (the immediate precursor of c-di-GMP), it can be used to determine the concentration of ATP, whose intracellular concentration closely correlate to GTP (Buckstein et al. 2008), and of other adenylic nucleotides.

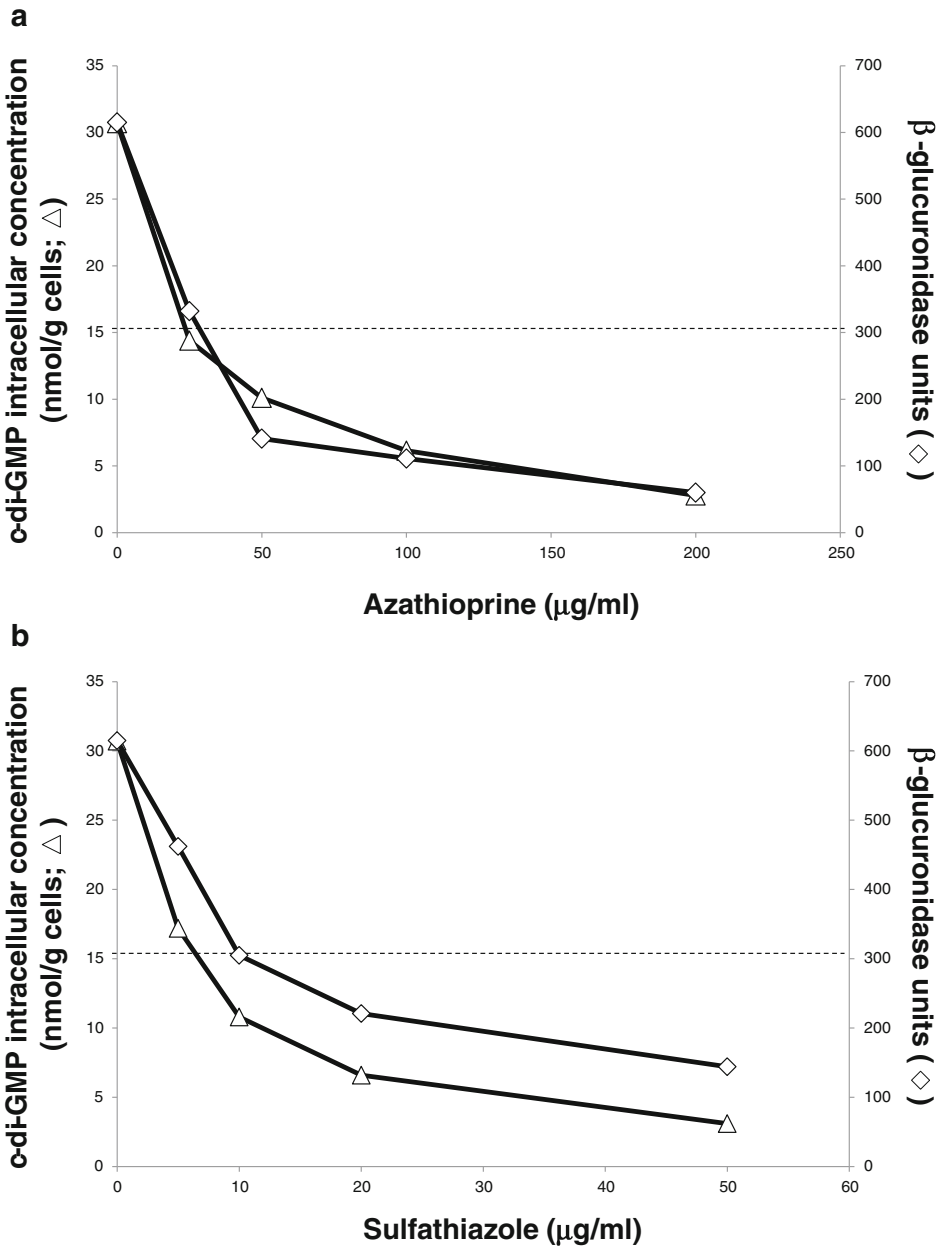

Fig. 2 Effects on WspR-dependent *csgBAC* gene expression and c-di-GMP production by azathioprine (**a**) and sulfathiazole (**b**). *Diamonds*, β-glucuronidase assays performed in the PHL856 (*csgA*::*uidA*-*kan*) strain transformed with pTOPOWspR. Average β-glucuronidase value in the absence of inhibitors was 615 units. The values are the average of at least three independent experiments: standard deviations were lower than 5 %. *Triangles*, inhibition of c-di-GMP biosynthesis in PHL565W/pTOPOWspR. In the control samples, c-di-GMP amounts were 30.7 nmol/g dry weight. Each value is the average of two independent experiments with very similar results. Values equal to 50 % of the untreated control are indicated by a *dashed line*

As shown in Fig. S2 and Table S2 in the ESM, intracellular ATP and ADP were only reduced by about 30 % at the highest azathioprine concentration tested (200 μg/ml), while a 42 % reduction was observed for adenosine. The observation that adenylic nucleotide pools are only slightly inhibited is in line with the lack of significant bacterial growth inhibition by azathioprine and seems to suggest a specific effect of this drug on c-di-GMP biosynthesis. Azathioprine was also able to inhibit c-di-GMP production catalyzed by overexpression of YdaM, a DGC important for curli production in *E. coli* (Weber et al. 2006), albeit at a slightly higher concentration ($IC_{50\ \%}$= 75 μg/ml (270 μM); Fig. S3 in the ESM), suggesting that its effect is not restricted to WspR.

Sulfathiazole, which also inhibits WspR-dependent curli production (Fig. 1b), was already shown to be an inhibitor of c-di-GMP biosynthesis in *E. coli* cells overexpressing the DGC proteins YdaM and AdrA (Antoniani et al. 2010). Consistent with these observations, sulfathiazole inhibited by 50 % both stimulation of *csgBAC* transcription and c-di-GMP biosynthesis by WspR at ca. 10 μg/ml (40 μM) (Fig. 2b).

DGC activity is not inhibited by antimetabolic drugs in enzymatic assays in vitro. Our results strongly suggest that both sulfathiazole and azathioprine can impair curli production and *csgBAC* operon transcription levels by interfering with c-di-GMP production by WspR (Figs. 1 and 2). Thus,

we tested whether sulfathiazole and azathioprine can act as direct inhibitors of DGC enzymatic activity by in vitro assays with purified proteins. Since azathioprine is thought to be a pro-drug, which is converted in 6-mercaptopurine and in its nucleotide derivatives *in vivo*, we also tested 2-amino-6-mercapto-purine riboside, a product of azathioprine metabolism. Initially, we tested the three compounds on the PleD protein from *C. crescentus* since this protein was the first DGC characterized for its structural and kinetic features in vitro and thus it represents a standard for DGCs (Paul et al. 2007; Wassmann et al. 2007). Azathioprine only showed weak inhibition (roughly 15 %) of PleD DGC activity at 500 μM, i.e., at more than 5-fold higher concentrations than what needed to inhibit c-di-GMP production by 50 % in the bacterial cell (Fig. 2a), while no detectable inhibition of PleD activity was observed in the presence of 2-amino-6-mercapto-purine riboside up to 500 μM (Fig. 3). Similarly, sulfathiazole (500 μM) did not show any significant inhibition of PleD enzymatic activity (Fig. 3).

Lack of inhibitory activity on PleD would suggest that the compounds tested are not specific inhibitors of DGCs. However, to verify this further, we purified the WspR protein and performed DGC assays in the presence of the molecules identified in our in vivo screening. DGC assays with WspR confirmed the results obtained on the PleD protein: none of the three compounds tested in enzymatic assays showed significant inhibition of DGC activity by WspR at 500 μM (Fig. 3). These results strongly suggest that inhibition of c-di-GMP biosynthesis in bacterial cells by azathioprine and sulfathiazole is indirect and not due to direct binding to DGC proteins.

DGC activity is affected by intracellular nucleotide availability in vivo Both sulfathiazole and azathioprine, the molecules found in our screening for DGC inhibitors, are antimetabolite drugs. Sulfathiazole is an inhibitor of folic acid biosynthesis, while azathioprine was shown to be an inhibitor of eukaryotic 5-aminoimidazole-4-carboxamide ribotide (AICAR) transformylase (Ha et al. 1990), an enzyme involved in purine nucleotide biosynthesis that uses 10-formyl-tetrahydrofolate, a folic acid-derived molecule, as substrate. We performed *in silico* docking experiments which strongly suggested high affinity binding of azathioprine to the cyclohydrolase domain of the AICAR transformylase (predicted Ki≈0.23 μM; Fig. S4a in the ESM), in agreement with the role of azathioprine as inhibitor of this enzyme. Although in silico docking experiments were performed on the human protein, all amino acid residues potentially involved in azathioprine binding are strongly conserved in bacteria, including *E. coli* (Fig. S4b in the ESM). Mutants in the *purH* gene are auxotrophic for purines (Baba et al. 2006), consistent with the importance of AICAR transformylase in de novo purine synthesis; however, their growth is only slightly impaired in complex media, such as CRL medium used in our experiments, that provide exogenous nitrogenous bases to bacterial cells (Fig. S5 in the ESM). To confirm its role of inhibitor of AICAR transformylase, we tested the antimicrobial effects of azathioprine on *E. coli* MG1655 grown in M9/Glu medium, in which glucose is the sole carbon source and purines can only be synthesized via the de novo pathway. Unlike in CRL medium, in which no antimicrobial activity was detected up to 200 μg/ml, azathioprine inhibited bacterial growth at 32 μg/ml (115 μM; Table S3 in the ESM). These observations strongly suggest that exposure of bacterial cells to either sulfathiazole or azathioprine, leading either to folic acid depletion or AICAR transformylase inhibition, may negatively affect intracellular purine nucleotide concentrations.

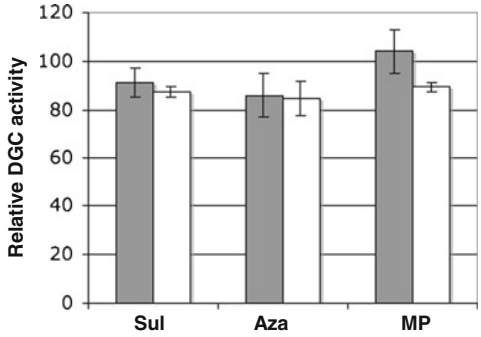

Fig. 3 Activity of purified DGCs in the presence of 500 μM sulfathiazole (*Sul*), azathioprine (*Aza*), and its metabolite 2-amino-6-mercapto-purine riboside (*6-MP*). *Grey and white bars* refer to PleD and WspR, respectively. The values refer to the percentage of residual activity after 1 h or 30 min of reaction, for PleD or WspR, respectively, considering the activity without inhibitor as 100 %. Enzymatic activities in the absence of inhibitors are: 43/38 nmol c-di-GMP min^{-1} mg^{-1} (PleD) and 24/38 nmol c-di-GMP min^{-1} mg^{-1} (WspR; ±5 % DMSO, respectively)

Interestingly, mutations resulting in alterations in the nucleotide biosynthetic pathways have been shown to impair biofilm formation and curli production (Ueda et al. 2009; Garavaglia et al. 2012): in particular, an *E. coli* MG1655 derivative carrying a deletion in *purH*, the gene encoding AICAR transformylase, is unable to produce curli, due to transcription downregulation of the *csg* operons (Garavaglia et al. 2012). WspR overexpression in a *purH* mutant derivative of PHL565W (PHL565WΔ*purH*::*cat*) failed to stimulate either curli or cellulose production, as determined by CR and CF binding and by biofilm formation assays (Fig. 4). Determination of intracellular c-di-GMP concentrations confirmed lack of WspR-dependent c-di-GMP biosynthesis in PHL565WΔ*purH*::*cat* (data not shown). Apparent lack of WspR DGC activity in a *purH* mutant background does not result from reduced levels of WspR protein production, as determined by SDS-PAGE analysis (Fig. S1 in the ESM).

Activity of antimetabolite drugs on E. coli clinical isolates Our results indicate that both azathioprine and sulfathiazole can

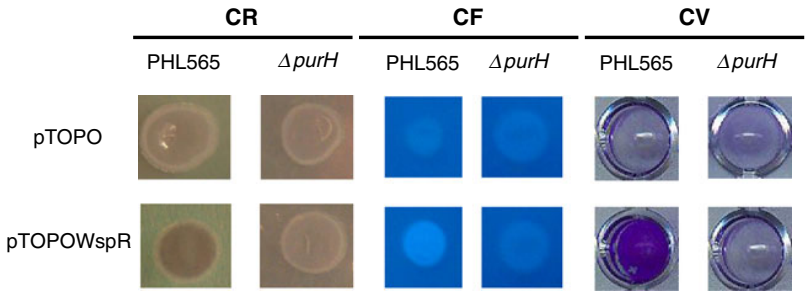

Fig. 4 Effects of WspR expression on CR binding and biofilm formation in the PHL565WΔ*purH*::*cat* strain in comparison to its reference strain. Semi-quantitative evaluation of biofilm in CV assays (calculated as in Materials and Methods) gave adhesion values of 1.6 for PHL565W/pTOPO, 14.8 for PHL565W/pTOPOWspR, 1.3 for PHL565WΔ*purH*::*cat* /pTOPO, and 1.24 for PHL565WΔ*purH*::*cat*/pTOPOWspR. Two independent experiments were performed, each time with samples repeated in triplicate. Standard deviations were lower than 10 % for all samples

prevent biofilm formation and inhibit c-di-GMP biosynthesis (Figs. 1 and 2); however, this activity was only measured in PHL565W/pTOPOWspR, i.e., in an *E. coli* laboratory strain expressing the *P. aeruginosa* DGC protein WspR. Thus, we tested whether azathioprine and sulfathiazole could be able to inhibit biofilm formation in *P. aeruginosa* and in *E. coli* isolates. Neither drug showed any significant activity on *P. aeruginosa* PAO1 biofilms (data not shown); in contrast, they strongly inhibited biofilm formation (Fig. 5) in two clinical isolates from Long-term care units responsible for haemolytic-uremic syndrome and urinary catheter-related infection, respectively. Unlike *E. coli* laboratory strains, in which curli production does not occur at 37 °C due to downregulation of the *csg* operons (Gualdi et al. 2008), both isolates showed red phenotype on CR medium at this temperature (Fig. 5). A red phenotype on CR medium at 37 °C suggests that the two isolates might be able to produce curli even during human host colonization, a rather common feature in pathogenic *E. coli* (Wang et al. 2010). The red phenotype was impaired, at least partially, both by azathioprine and by sulfathiazole. The azathioprine derivative 2-amino-6-mercapto-purine riboside was also tested on clinical isolates, showing efficient inhibition of biofilm formation and of CR red phenotype (Fig. 5).

Discussion

The signal molecule c-di-GMP promotes biofilm formation through stimulation of extracellular polysaccharides and cell surface-associated factors (Romling and Amikam 2006). c-di-GMP-mediated biofilm formation is an important factor in host colonization and appears to play a major role in chronic diseases such as lung infection in cystic fibrosis patients (Haussler 2004; Kulasakara et al. 2006; Tamayo et al. 2007; Cotter and Stibitz 2007). Thus, inhibition of DGCs, the enzymes responsible for c-di-GMP biosynthesis, might

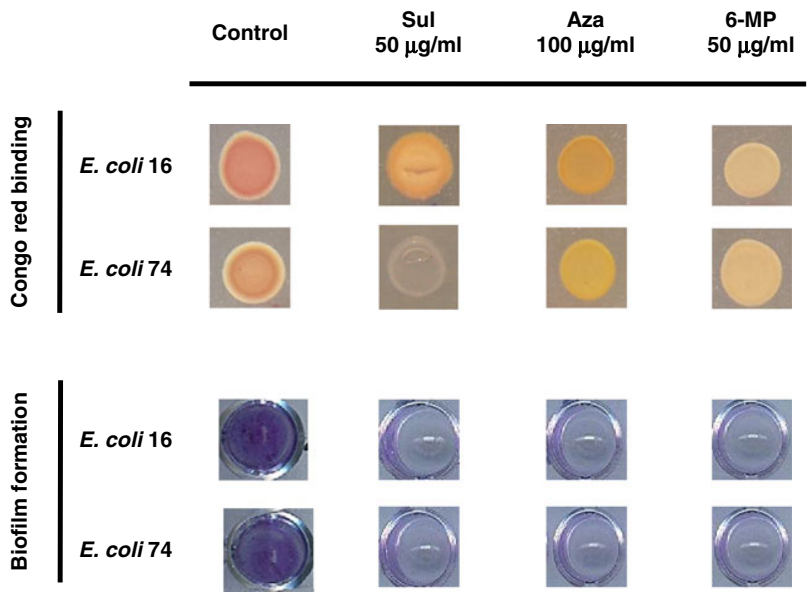

Fig. 5 CR binding and biofilm formation in clinical isolates of *E. coli*. Experiments were performed as in Fig. 1b, except that bacteria were grown at 37 °C. *Abbreviations: Sul* sulfathiazole, *Aza* azathioprine, *6-MP* 2-amino-6-mercapto-purine riboside

counteract host colonization by pathogenic bacteria and might complement antimicrobial therapies with conventional antibiotics. We had already identified the sulfonamide antibiotic sulfathiazole as an inhibitor of c-di-GMP synthesis (Antoniani et al. 2010). Through a screening strategy for inhibitors of the *P. aeruginosa* DGC WspR, based on microbiological assays in an *E. coli* reporter strain, we have now found that azathioprine can inhibit c-di-GMP biosynthesis (Fig. 2a). These molecules do not seem to inhibit DGCs directly, based on enzymatic assays performed with purified PleD and WspR proteins (Fig. 3), although we cannot rule out the possibility that metabolic derivatives of azathioprine other than 2-amino-6-mercapto-purine riboside might be enzymatic inhibitors of DGCs. Alternatively, both sulfathiazole and azathioprine could hamper c-di-GMP biosynthesis through their antimetabolite activity, i.e., by impairing de novo nucleotide biosynthesis in the bacterial cell, although our results suggest that, when bacterial cultures are grown in a complex medium able to provide an exogenous source for nucleotide biosynthesis, exposure to azathioprine only results in a partial inhibition of nucleotide pools (Fig. S2 in the ESM). However, it is possible that an even slight reduction in nucleotide availability, affecting intracellular concentrations of GTP, the substrate for DGCs, might result in a significant decrease in c-di-GMP biosynthesis: indeed, it is conceivable that GTP pools are channeled by the cell towards primary metabolism (e.g., transcription and translation) rather than to signal molecule production. Intracellular concentrations of nucleotides, and even ratios between concentrations of different intermediates in nucleotide biosynthesis, have been shown to affect production of c-di-GMP-dependent extracellular structures both in *P. aeruginosa* and in *E. coli* while not impacting primary metabolism (Ueda et al. 2009; Garavaglia et al. 2012; Attila et al. 2009), suggesting that feedback control of DGC activity by relatively small changes in nucleotide availability does indeed take place in bacteria. Thus, our results reiterate the relevance of drugs targeting nucleotide biosynthesis as potential antibiofilm agents acting through indirect inhibition of c-di-GMP biosynthesis.

Our observations that the anti-inflammatory drug azathioprine is an antibiofilm agent and an inhibitor of c-di-GMP biosynthesis in the bacterial cell (Figs. 1 and 2) might have important implication for the understanding of its mechanism of action. Azathioprine is widely used in treatment of both Crohn's disease and ulcerative colitis, in order to counteract chronic bowel inflammation typical of these syndromes. The anti-inflammatory activity by azathioprine relies on the blockade of CD28-dependent Rac1 activation in human CD4+ lymphocytes by one of its metabolites, 6-thioguanosine-triphosphate, ultimately leading to intestinal T cell apoptosis (Tiede et al. 2003). Current models point to a direct role of intestinal microbial flora in the development of inflammatory bowel diseases, suggesting that chronic inflammation results from an overly stimulated immune response of gut-associated lymphoid tissue (GALT) against components of the enteric flora (Kaser et al. 2010). Additionally, various extracellular structures are involved in the adhesion to intestinal epithelial cells of adherent invasive *E. coli* (AIEC) strains, which can be found in increased proportions in the *lamina propria* of patients with ileal Crohn's disease, and are suspected to contribute to disease pathogenesis (Darfeuille-Michaud et al. 2004; Baumgart et al. 2007). Although azathioprine possesses antimicrobial activity against *Mycobacterium avium* subspecies *paratubercolosis* (Shin and Collins 2008; Krishnan et al. 2009), a bacterium linked to this pathology (Hermon-Taylor 2009), microbial growth inhibition by azathioprine did not seem to play a major role in its anti-inflammatory effects, due to its very narrow spectrum of activity. However, our results indicate that azathioprine, and its derivative 2-amino-6-mercapto-purine riboside, can prevent c-di-GMP biosynthesis, thus hampering production of bacterial extracellular structures (Figs. 1, 2, 3, 4, and 5). Inhibition of c-di-GMP biosynthesis may thus lower the antigenic load to the GALT, ultimately reducing detrimental immune responses. Indeed, in addition to controlling the synthesis of various highly antigenic extracellular structures, such as polysaccharides, curli, and other adhesins (Romling and Amikam 2006; Cotter and Stibitz 2007), c-di-GMP itself is a powerful effector of the human immune response (Karaolis et al. 2007; Gray et al. 2012). c-di-GMP might be released by bacterial cells lysed by neutrophils at an infection site, and reach significant local concentrations, eliciting a strong response by the immune system. Inhibition of c-di-GMP synthesis in bacteria might tone down this immunostimulatory effect, relieving inflammation.

Similar to azathioprine, the sulfonamide antibiotic sulfathiazole also appears to inhibit c-di-GMP biosynthesis through its antimetabolite activity (Antoniani et al. 2010; Figs. 1, 3, 4, and 5). It is noteworthy that methotrexate, which, like sulfonamides, targets folic acid biosynthesis, is also used as an anti-inflammatory drug both in Crohn's disease and in autoimmune pathologies such as rheumatoid arthritis (Absah and Faubion 2012; Deighton et al. 2009). Another drug used in various pathologies associated with chronic inflammation, sulfasalazine, is a combination of the anti-inflammatory compound 5-aminosalicylic acid with the sulfonamide antibiotic sulfopyridine: recent clinical studies indicate that sulfasalazine is more effective for remission of Crohn's disease than any other 5-aminosalicylic acid-based drug (Levesque and Kane 2011), thus suggesting that the sulfonamide moiety might be essential for its effectiveness. Full understanding of the role of antimetabolite drugs in modulating synthesis of c-di-GMP, and possibly of other bacterial signal molecules, could be crucial to improve their efficacy not only in the treatment of bacterial infections but also of chronic inflammatory diseases with complex etiology in which host colonization by bacterial biofilm can represent a component.

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SUPPLEMENTARY TABLES

Table S1. List of polypeptides identified by MALDI-TOF analysis after in-gel trypsin digestion (SDS-PAGE shown in Figure S1). Only the three proteins showing the highest scores are listed for each band.

Lane (Bacterial strain)	NCBI gene accession number and name of identified protein	MW (Da)	Number of peptides and %coverage	Score
1 (PHL565W/ pTOPO)	gi|147379 Phosphoribosylpyrophosphate synthetase [*Escherichia coli*]	34437	3 in amino acid region 94-356; 12%	163
	gi|15799867 Acetyl-CoA carboxylase carboxyltransferase subunit alpha [*Escherichia coli*]	35333	3 in amino acid region 94-381; 15%	147
	gi|15800816 Outer membrane protein A [*Escherichia coli*]	37292	3 in amino acid region 10-205; 14%	121
2 (PHL565W/ pTOPOWspR)	gi|15598897 Two-component response regulator, GGDEF protein WspR [*Pseudomonas aeruginosa*]	37977	18 in amino acid region 9-347; 60%	1309
	gi|147379 Phosphoribosylpyrophosphate synthetase [*Escherichia coli*]	34437	4 in amino acid region 90-277; 16%	245
	gi|147464 Aspartate transcarbamoylase catalytic chain (*pyrB*) [*Escherichia coli*]	34464	3 in amino acid region 115-366; 12%	164

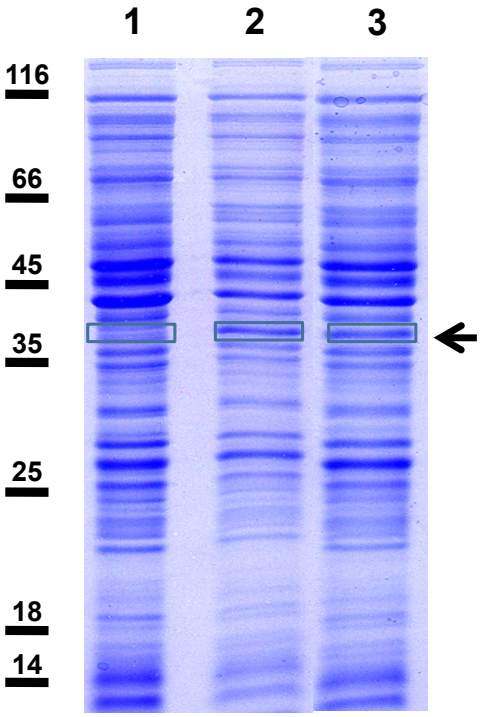
Table S2. Intracellular levels of adenylic nucleotides as determined by HPLC analysis shown in Fig. S2.

	pmol/ml culture		
	ATP	**ADP**	**Adenosine**
Control	**630**	**77**	**102**
Aza 100 ug/ml	**447**	**48**	**75**
Aza 200 ug/ml	**435**	**58**	**59**

Table S3. Antimicrobial activity (MIC in mg/ml) by azathioprine on *E. coli* strains in both CRL (complex) and M9/Glu (minimal) medium.

***E. coli* strains**	**CRL**	**M9/Glu**
MG1655	**>128**	**32**
PHL565W/pTOPO	**>128**	**32**
PHL565W/pTOPOWspR	**>128**	**32**
Clinical isolate 16	**>128**	**64**
Clinical isolate 74	**>128**	**64**

SUPPLEMENTARY FIGURES



Supplementary Figure 1

Figure S1. SDS-PAGE showing WspR expression levels in PHL565W/pTOPOWspR (Lane 2) and in PHL565WΔ*purH::cat*/pTOPOWspR (Lane 3). The band at ca. 38 KDa corresponding to the WspR protein is indicated by an arrow. Cell extract of PHL565W harboring the control vector pTOPO is shown in Lane 1. The position of molecular weight markers is shown by the ticks on the left. Squares indicate the sections of the gel excised for MALDI-TOF analysis (See Table S1).

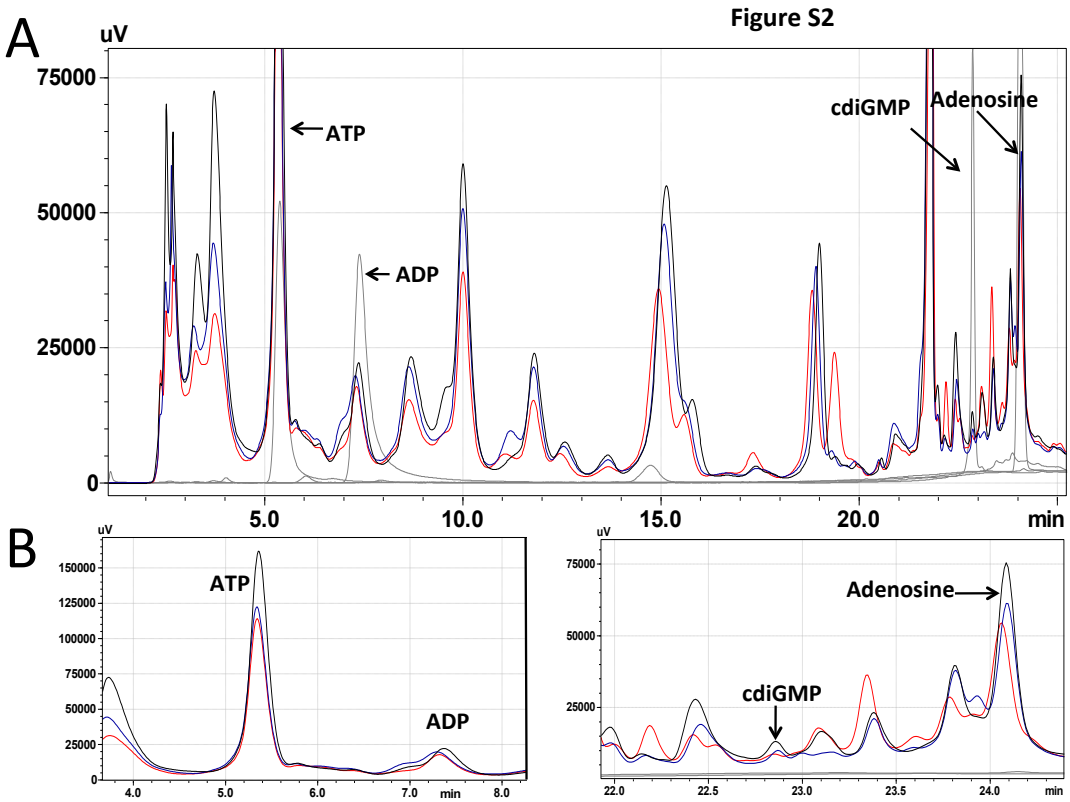


Figure S2. A HPLC chromatograms of nucleotide extracts prepared from overnight cultures of bacterial cells grown in the absence (black line) and in the presence of 100 µg/ml (blue line) and 200 µg/ml azathioprine (red line). For peak identification, c-di-GMP and adenylic nucleotides standards were analyzed under the same elution conditions (gray lines). **B** Magnification of chromatograms' sections showing peaks corresponding to ATP, ADP, adenosine and c-di-GMP.

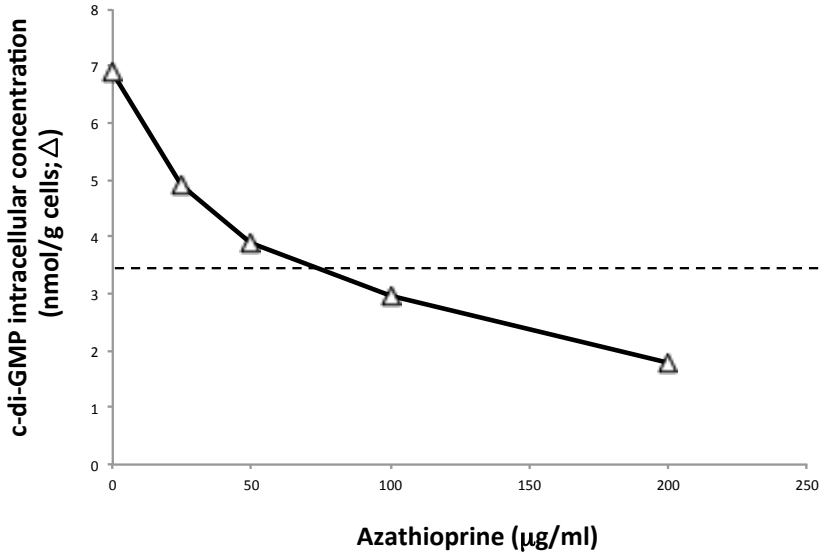


Fig. S3

Figure S3. Inhibition of c-di-GMP biosynthesis by azathioprine in the *E. coli* strain PHL565W/pTOPOYdaM. In the control samples, c-di-GMP amounts were 6.9 nmol/g dry weight. Data shown are the average of two independent experiments with very similar results. Values equal to 50% of the untreated control are indicated by the dashed line.

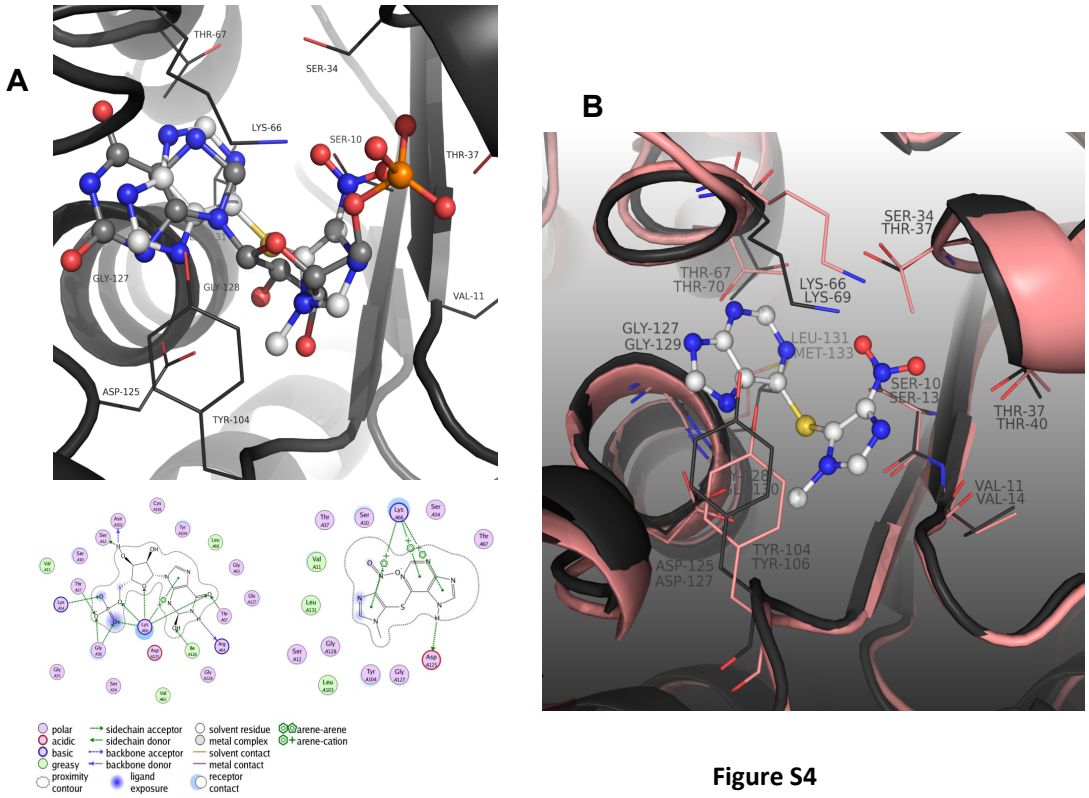


Figure S4

Figure S4. A Docking simulation of the interaction between the binding cleft of the cyclohydrolase domain of human enzyme Aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase/IMP cyclohydrolase (shown as black carbon cartoon) and azathioprine (white carbon). Nitrogen atoms are shown in blue, oxygen in red, phosphate orange. Xanthosine-5'-monophosphate (XMP) is shown as reference. A schematic interaction diagram between the enzyme and XMP (left) or azathioprine (right) is shown below. **B** Comparison between the binding cleft of the cyclohydrolase domain of human (shown as black carbon cartoon) and *E. coli* (shown as pink carbon cartoon) enzymes. Nitrogen atoms are shown in blue, oxygen in red, phosphate orange. Upper label residue numbering refers to the human enzyme (upper label) and lower label residue numbering refers to the *E. coli* enzyme

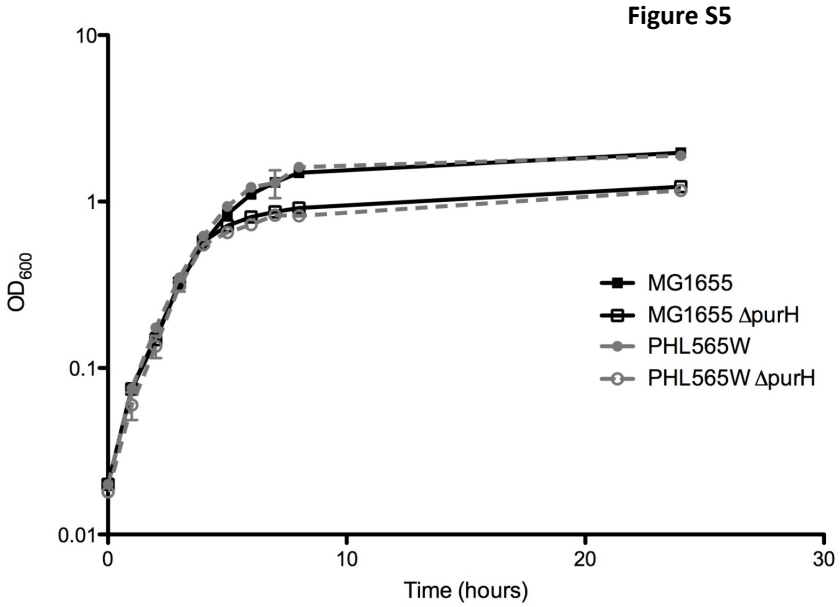


Figure S5. Effect of the *purH* mutation on growth in CRL medium. Bacteria were grown at 30°C and turbidity was measured using a spectrophotometer as OD600nm. Squares: MG1655 strain; circles: PHL565W strain. Open symbols: *purH* mutant allele; closed symbols: *purH* wild type allele.

PART III

Content

Manuscript in preparation:

Rossi E. and Landini P. Phosphoadenosine 5'-phosphosulphate (PAPS) levels affect production of curli fibres and other extracellular factors in *Escherichia coli*

Phosphoadenosine 5'-phosphosulphate (PAPS) levels affect production of curli fibres and other extracellular factors in *Escherichia coli*

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Running Title: Curli fibres production is stimulated upon phosphoadenosine 5'-phosphosulphate accumulation

Abstract

In response to different environmental and physiological signals, bacteria synthesize a wide variety of extracellular structures, able to mediate cell-cell and cell-surface interactions and to promote development of bacterial biofilms. We show that, in *Escherichia coli*, inactivation of the *cysH* gene, encoding phosphoadenosine 5'-phosphosulfate (PAPS) reductase, strongly affects cell surface-associated structures. In particular, inactivation of the *cysH* gene stimulates production of curli fibres (one of the main adhesion factors in *E. coli*) through regulation of the *csgBAC* operon, encoding curli structural subunits, at mRNA stability level. Activation of curli production and *csgBAC* expression seems to depend on PAPS accumulation, since mutations in the sulphate assimilation pathway, to which *cysH* belongs, do not show comparable effects. In addition to curli, the *cysH* mutation affects the production of outer membrane proteins Antigen 43, OmpX and Slp, as well as one or more extracellular polysaccharides. We speculate that perturbation of intracellular PAPS levels allow the bacterial cell to sense environmental sulphur availability and to trigger changes in bacterial physiology which involve increased production of cell aggregation factors.

Introduction

In natural environments, bacteria are exposed to highly dynamic and complex conditions. A quick and specific response to different stimuli is required for adaptation and survival. Such adaptation is achieved synthetizing a wide variety of extracellular surface structures, which include curli, pili, flagella and extracellular polysaccharides (EPS), such as cellulose and poly-*N*-acetylglucosamine (PNAG). These structures fulfil different duties facilitating cellular binding to biotic and abiotic surfaces, motility and molecules trafficking.

In *Escherichia coli* and other members of *Enterobacteriaceae*, curli amyloid fibres bind Congo red (CR) dye and mediate cell aggregation, cell-to-surface adhesion, and more in general are associated with biofilm formation (Hammar et al., 1995; Cookson et al., 2002; Kikuchi et al., 2005). Two divergently transcribed operons are required for curli production: the *csgBAC* operon, which encodes for the two structural subunits, and the *csgDEFG* operon, from which are expressed the transcriptional regulator CsgD and proteins required for curli export and assembly (Barnhart and Chapman, 2006). Curli biosynthesis regulation is extraordinarily complex and responds to a combination of environmental cues (Gerstel and Römling, 2003), such as, low temperature, low osmolarity, slow growth (Römling et al., 1998; Lacour and Landini, 2004), and oxygen availability (Tagliabue, Maciag, et al., 2010; Gerstel and Römling, 2001). A number of regulators, including RpoS, OmpR, IHF, H-NS, CpxR, Crl, MlrA, has been proved to actively regulate the expression of the *csgD* promoter (Römling et al., 1998; Prigent-Combaret et al., 2001; Hung et al., 2001; Gerstel et al., 2004; Bougdour et al., 2004; Brown et al., 2001).

Curli expression is also strongly dependent on the signal molecule c-di-GMP; indeed, curli transcription is controlled by the activity of at least three c-di-GMP control modules (YegE/YhjH, YdaM/YciR, DosC/DosP), each made up of one digunylate cyclase (DGC), which synthetize the cyclic nucleotide and one phosphodiesterase (PDE) which degrades it (Pesavento et al., 2008; Tagliabue, Maciag, et al., 2010; Lindenberg et al., 2013). In *E. coli*, c-di-GMP also modulates the expression of other structures involved in biofilm formation: cellulose production depends on the activity of the two DGCs, AdrA and YedQ (Römling et al., 2000; Da Re and Ghigo, 2006), while PNAG biosynthesis is under the direct control of the DosP/DosC proteins (Tagliabue, Antoniani, et al., 2010). More in general, c-di-GMP

plays a pivotal role in the transition between planktonic cells and sessile communities in many Gram-negative bacteria, inducing the production of adhesion factors and EPS, such as PEL and PSL exopolisaccarides in *P. aeruginosa* (Hickman et al., 2005), and type I fimbriae, cellulose and another EPS in *B. cenocepacia* (Fazli et al., 2011, 2013).

In addition to dedicated signal molecules, metabolic intermediates or products can also affect extracellular structures production: for instance, in *E. coli*, expression of genes coding for curli fibres is modulated in response to glycolysis intermediates, *N*-acetylglucosamine-6-phosphate and nucleotides levels (Reshamwala and Noronha, 2011; Barnhart et al., 2006; Garavaglia et al., 2012). Likewise, *de novo* UMP biosynthesis is necessary for the production of biofilm determinants in *P. aeruginosa* (Attila et al., 2009; Ueda et al., 2009). In addition, other metabolic products has been connected with the regulation of surface exposed structures: indole, a product of tryptophan degradation, stimulates EPS production in *V. cholerae* (Mueller et al., 2009), while in *B. subtilis* D-amino acids promote the release of the TasA adhesin from the cell wall triggering biofilm dispersal (Kolodkin-Gal et al., 2010).

In the present study, we show that levels of phosphoadenosine 5'-phosphosulphate, an intermediate of the sulphate reduction for cysteine/methionine biosynthesis pathway, regulate the production of curli fibres and other extracellular structures including a yet unknown exopolysaccharide. In particular we show that PAPS regulates curli expression at level of mRNA stability, indicating that the molecules could affect RNA degradation of specific transcripts modulating the reshaping of the cellular surface in response to sulphur availability in the environment.

Materials and Methods

Bacterial strains and growth condition

Bacterial strains used in this work are listed in Table 1. For strain construction and manipulation, bacteria were grown in LB medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl).

For Congo red (CR) and Calcofluor (CF) binding assays, overnight cultures were spotted, using a replicator, on either (CR)-supplemented or (CF)-supplemented agar media. Both media are composed by 1% Casamino acids, 0.15% yeast extract, 0.005% $MgSO_4$, 0.0005% $MnCl_2$, 2% agar to which 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 20 h at 30°C and 37°C; phenotypes were better detectable after 24-48 h incubation at 4°C.

For gene expression regulation studies bacteria were grown in liquid medium composed by 1% Casamino acids, 0.15% yeast extract, 0.005% $MgSO_4$, 0.0005 $MnCl_2$. Although Congo red is not present, this medium was called "liquid Congo red" medium (CRL), due to its identical composition as the CR medium utilized for Congo red binding assays.

For testing cysteine auxotrophy and growth in presence of organosulphonates bacteria were grown from 24 to 48 hours at 37°C either in M9 or sulphate-free M9 (SF-M9) media with or without supplementation either with 0.25 mM cysteine or with 0.25 mM taurine. Sulphate-free medium (SF-M9) was obtained substituting $MgSO_4$ with $MgCl_2$ at a final concentration of 1 mM. Unless otherwise stated, 0.4% glucose was added as sole carbon source to give M9 Glu or SF-M9 Glu media.

If required, antibiotics were added at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 50 μg/ml; kanamycin, 50 μg/ml; tetracycline, 25 μg/ml; rifampicin, 100 μg/ml.

Plasmid construction

Plasmids and primers used in this work are listed in Table 1 and Supplementary Table S1. For expression of wild-type CysH protein, the corresponding gene was amplified by polymerase chain reaction (PCR) from *E. coli* MG1655 chromosome using primer pair cysH_NdeI_for/cysH_PstI_rev, and the resulting product was cloned into pT7-7 vector using the NdeI/PstI restriction sites. The pT7cysH$_{mut}$ plasmid, carrying a

mutated allele of the *cysH* gene in which the redox-active centre (Berendt et al., 1995) is inactivated, was constructed amplifying the *cysH* gene using the primer pairs cysH_NdeI_for/cysH_mut_PstI_rev resulting in the following substitutions: T → G at nucleotide 715, G → C at nucleotide 716, C → G at nucleotide 724, A → C at nucleotide 725. The four mutations result in substitution of Cysteine 240 and Histidine 243 to Alanine residues (E**C**GL**H** → E**A**GL**A**). The obtained PCR product was cloned into pT7-7 vector as described for the wild type allele using the NdeI/PstI restriction sites. Both the wild type and mutant alleles of the *cysH* gene were verified by sequencing.

Genetic techniques

Transposon insertion mutagenesis was carried out using the EZ-Tn5<R6KYori/KAN-2> transposome (Epicentre). Transposon mutagenesis and determination of transposon insertion site by rescue cloning were carried out according to manufacturer's instructions. *E. coli* MG1655 mutant derivatives were constructed using λ Red technique (Datsenko and Wanner, 2000). The list of primers used for gene inactivation and for confirmation of target gene disruption by polymerase chain reaction (PCR) is presented in Supplementary Table S1.

Gene expression assays

Gene expression levels were measured through quantitative (real time) reverse transcriptase PCR (quantitative RT-PCR) as previously described (Gualdi et al, 2007), using 16S RNA as a reference gene. RNA was extracted from overnight cultures grown in CRL medium at 30°C or 37°C in full aeration, achieved by constant shaking at 100 rpm/minute. The complete list of primer used for amplification is reported in Supplementary Table S1. mRNA half-lives were measured by RT-PCR experiments in the presence of rifampicin as previously described (Wang et al., 2005).

Other techniques

Biofilm formation was determined with the surface attachment assay (Gualdi et al., 2008) performed on bacterial cultures grown overnight in CRL at 30°C and 37°C. For proteomic analysis outer membrane proteins (OMPs) enriched samples were obtained using the N-lauroylsarcosinate method as previously described (Gualdi et al., 2007).

Enriched samples were treated with RapiGest^{TM} SF (Waters Corporation) and then trypsin digested as described (Peano et al., 2014). Digested samples were then analysed by two-dimensional micro-liquid chromatography coupled to ion trap mass spectrometry (2DC-MS/MS) using ProteomeX-2 configuration (Thermo Electron Corporation, San Josè, CA, USA), according to (Palma et al., 2010). The experimental mass spectra produced by MudPIT analyses were correlated to tryptic peptide sequences by comparing with theoretical mass spectra, obtained by *in silico* digestion of *E. coli* K-12 str. MG1655 protein database. Analysis output were treated with an in-house software called MAProMa (Multidimensional Algorithm Protein Map) (Mauri and Dehò, 2008) to identify differentially expressed proteins (DEPs). Differentially expressed proteins are estimated by means of two algorithms of MAProMa, DAve (Differential Average), measuring changes in expression levels, and DCI (Differential Coefficient Index), measuring total protein amount in a given sample (Mauri et al., 2005). A DAve value either >0.1 or <-0.1 is an indication of different relative expression level between two samples, while a DAve value either = 200 or = -200 indicates exclusive presence of a protein in only one sample.

Statistical analysis

If not otherwise stated, all experiments were performed at least in triplicate and the data were analysed by one-way analysis of variance, with a *p*-value of 0.05 being significant, using the statistical software package R (R Core Team, 2013).

Results

Inactivation of the *cysH* gene affects the production of curli fibres and of other extracellular structures. Congo red and Calcofluor dyes provide an easy way to identify the production of surface exposed structures of both proteinaceous and exopolysaccharidic nature; Congo red (CR) binds with high affinity to amyloid fibres, such as curli fibres (Hammar et al., 1995) and with lower affinity to cellulose (Teather and Wood, 1982) and poly-*N*-acetylglucosamine (Romeo, 1998); Calcofluor (CF) binds cellulose and chitin (Harrington and Raper, 1968) but also react with other polysaccharides (Herth and Schnepf, 1980). Curli fibres represent the main extracellular structure bound by CR dye in *E. coli* MG1655 at 30°C; no curli or other CR-binding extracellular structures are synthesized at temperatures higher than 32°C in this strain (Olsén et al., 1993; Figure 1A).

To identify novel genes involved in extracellular structures regulation, we carried out transposon mutagenesis in the *E. coli* K-12 strain MG1655; mutants were screened for their phenotype on CR and CF medium both at 30°C and 37°C, to search for genes involved in the temperature regulation of curli fibres. We isolated several mutants showing altered phenotypes on both media (data not shown). One mutant displaying a dark red phenotype at 30°C and a red coloration at 37°C on CR, and an increased fluorescence at both temperature on CF was further characterized (Figure 1A).

Mapping of the Tn5<R6Kγ*ori*/KAN-2> transposon indicated that the insertion site lay at nucleotide 472 of the "735 nucleotide-long" *cysH* gene, encoding 3'-phospho-adenylylsulfate reductase enzyme (PAPS reductase). PAPS reductase catalyses the conversion of phosphoadenosine-5'-phosphosulphate (PAPS) into adenosine 3',5'-bisphospate (pAp) and sulphite, in the sulphate reduction pathway leading to sulphur assimilation and cysteine biosynthesis (Figure 2A). The location of the transposon insertion suggested inactivation of the cysH gene: to verify this hypothesis, we constructed a *cysH* mutant in which the complete coding sequence of the gene was deleted (MG1655Δ*cysH*). As expected, this mutant displayed the same phenotypes on CR and CF as the MG1655*cysH::Tn5-kan* strain (Figure 2B) and was therefore used in further experiments.

The Δ*cysH* phenotype on CR suggested an increased production of curli fibres, while CF fluorescence hinted to the possible induction of the synthesis of an

extracellular polysaccharide. Production of adhesion factor, such as curli fibres, affects cellular aggregation and adhesion on solid surfaces (Cookson et al., 2002; Vidal et al., 1998), therefore we tested the mutant for its ability to adhere to an abiotic surface. Microtiter adhesion assay revealed a slight (2-fold), but highly reproducible increase in biofilm formation by the MG1655Δ*cysH* strain at 30°C (Figure 1B), while no significant differences between strains were observed at 37°C (data not shown). Confocal microscopy observations provided further confirmation that *cysH* inactivation stimulates the production of aggregative structures (Fig 1C). The effect of the mutation seems to be stronger at 30°C, indeed, at this temperature the mutant strain showed the formation of bigger and higher in number cellular aggregates (Figure 1C). At 37°C no aggregates where detectable in the wild-type strain, while only a few were found in the mutant strain (data not shown). Once again, these data suggest that the mutation affects the production of aggregative factors mainly expressed at low temperature, *i.e.* curli fibres. To verify that changes in phenotypes on CR and in the adhesion ability observed in the MG1655Δ*cysH* strain depended on curli production, we constructed a Δ*cysH* strain unable to synthesize this specific adhesion factor: as expected, the MG1655Δ*cysH*Δ*csgA* double mutant lost its ability to adhere to the microtiter (Figure 1B), but it still displayed a red phenotype on CR at both 30°C and 37°C (Figure 1A). When compared to its reference strain MG1655Δ*csgA*, the MG1655Δ*cysH*Δ*csgA* double mutant still displayed cellular aggregates at 30°C, although smaller than the MG1655Δ*cysH* mutant (data not shown). These result suggest that the *cysH* mutation, in addition to promoting curli production at 30°C, also stimulates the production of another extracellular factor able to bind CR and CF at both temperatures and to act as a weak cell aggregation factor. Because CF binds to EPS with very high affinity, MG1655Δ*cysH*Δ*csgA* phenotypes strongly suggested that *cysH* inactivation might influence the production of exopolysaccharides. Therefore, we inactivated in a Δ*cysH* background the genes *bcsA, wcaD*, *wzyE*, *pgaA* required respectively for the production of cellulose, colanic acid (CA), enter common antigen (ECA) and poly-*N*-acetylglucosamine (PNAG), that represent four of the most common *E. coli* EPS (Figure S1) (Stevenson et al., 1996; Rahman et al., 2001; Zogaj et al., 2001; Itoh et al., 2008). However, all the mutants showed the same phenotypes of the MG1655Δ*cysH*, suggesting that *cysH* mutation could either affect a different polysaccharide from the tested ones or it could induce the production of multiple EPS at the same time.

Mutants' effects depend on accumulation of the pathway intermediate PAPS. PAPS reductase belongs to the sulphate reduction pathway necessary for the biosynthesis of cysteine in *Escherichia coli* (Kredich, 1996). Indeed, both transposon insertion and deletion of the *cysH* gene led, as expected, to cysteine auxotrophy, as showed by their inability to grow in M9 Glu minimal medium (Supl. Table S2). Addition of cysteine at 0.25 mM to the medium fully overcomes the growth defect (Supl. Table S2). We wondered whether the effect of the *cysH* mutation on cell surface-associated structures could be associated to PAPS accumulation or to cysteine depletion. Indeed, starvation for various nutrients represents an important cue for the induction of the biosynthesis of extracellular structures involved in cellular aggregation and biofilm formation (Gerstel and Römling, 2001). To address this point, we checked for MG1655Δ*cysH* growth defect in the Casamino acid based liquid medium (CRL), which had the same composition of the medium used for the preparation of CR/CF agar plates. The mutant showed a growth rate similar to the wild-type strain, suggesting that the medium contained enough cysteine to sustain a normal growth (Figure S2). Hence, we concluded that in our test condition the mutant was not subjected to starvation for cysteine and that the effects observed were indeed due to the sole inactivation of the *cysH* gene.

As further verification that cysteine starvation was not responsible for the effects on CR/CF plates and in adhesion and aggregation experiments and to investigate whether the effects of *cysH* inactivation could also be observed for other genes belonging to the sulphate reduction pathway, we constructed knock out mutants in the *cysU*, *cysD, cysC* and *cysI* genes. All the mutants showed no growth in M9 Glu medium unless cysteine was added (Supl. Table S2), but when tested on CR and CF plates they showed wild type phenotypes (Figure 2B), ruling out the possibility that *cysH* inactivation effects were due to cysteine starvation. These results strongly suggest that the phenotypes observed in the MG1655Δ*cysH* strain are due to lack of the CysH protein. To verify that the effects of the *cysH* mutation were indeed connected to the PAPS reductase activity of the protein, we generated a mutant allele of the *cysH* gene in which we substituted the codons encoding two C-term amino acid identified as the redox-active centre of the enzyme (Berendt et al., 1995), with codons encoding for two alanine residues and cloned it in the pT7-7 vector (pT7$cysH_{mut}$). We then compared the effect of the mutant allele to the effects of the wild type one

(pT7cysH$_{wt}$) on MG1655Δ*cysH* phenotypes. As expected, the mutant allele was not able to complement the growth defect of the strain in M9 Glu medium (Supl. Table S2), confirming its inability to carry out PAPS reductase activity. As shown in Figure 2C, only the wild type allele was able to rescue the mutant phenotypes, confirming that CR and CF phenotypes were due to the lack of PAPS reductase activity of the CysH protein. In *cysH* mutants phosphoadenosine 5'-phosphosulfate is not converted to sulphite and pAp, causing PAPS to accumulate in cell. Thus, our observations suggest that effects of the *cysH* gene inactivation depend on the intracellular level of the modified nucleotide PAPS.

PAPS accumulation affects curli production acting on the stability of the *csgBAC* mRNA. Since our results suggested that PAPS accumulation could affect curli production, we investigated its effects on the expression of curli-related genes. Curli biosynthesis is under the control of a complex regulatory network, targeting in particular transcription initiation at the *csgDEFG* promoter (Römling et al., 2000; Gerstel and Römling, 2003; Gerstel et al., 2004). Thus, by quantitative Real Time PCR, we determined the transcript levels of the *csgD* gene, encoding the curli transcriptional regulator CsgD, the *csgF* gene, which also belongs to the *csgDEFG* operon and is involved in curli subunit export and assembly, and *csgB*, coding for the curli minor structural subunit and part of the *csgBAC* operon. Finally, we tested *adrA*, whose product is a diguanylate cyclase involved in cellulose production that, like *csgB*, is under direct control of the CsgD transcriptional regulator (Römling et al., 2000). As shown in Figure 3A, *cysH* inactivation resulted in a drastic increase in *csgB* expression (~30-fold induction), while showed a much more reduced effect on the *csgD* gene (~1.7-fold induction). Surprisingly, a more significant increase in *csgF* transcript levels was observed (~ 4-fold increase; Figure 3A), suggesting that, although belonging to the same operon, *csgD* and *csgF* might be subjected to a different forms of regulation in the MG1655Δ*cysH* mutant. Transcription of the CsgD-dependent gene *adrA* was unaffected in the MG1655Δ*cysH* strain (Figure 3A), consistent with wild-type expression levels of *csgD*. Thus, RT-PCR experiments suggest that the *cysH* mutation and PAPS accumulation mainly affect the *csgBAC* operon transcript levels, while not changing the expression of the *csgD* gene and having a minor effect on the *csgF* gene. Overexpression of the *cysH* gene from the pT7cysH$_{wt}$ plasmid in a wild type background further confirmed this result, showing a

significant downregulation (5-fold reduction) of the *csgB* gene, while leaving *csgD* and *adrA* transcript levels unaffected (Figure 3B). We also tested *csgB* expression levels in CRL medium supplemented with 0.25mM cysteine, and observed a 14-fold induction in the MG1655Δ*cysH* strain (data not shown), a further confirmation that the effect of the *cysH* mutation is not due to cysteine depletion.

Although *csgD* level in the MG1655Δ*cysH* strain showed a 1.7-fold increase (Figure 3A), it seems unlikely that such a small induction can account for the much larger effect observed on the *csgBAC* operon. Furthermore, *cysH* inactivation did not lead to any activation of the CsgD-dependent *adrA* gene, suggesting that *csgB* up-regulation might be independent of CsgD. As CsgD represents the main transcriptional regulator acting on the *csgBAC* operon, we hypothesized that higher transcript levels could be due to a change in mRNA stability. Indeed, mRNA decay experiments (Figure 3C) revealed a striking change in the mRNA stability of the *csgB* transcript in the MG1655Δ*cysH* strain, with its half-life increasing from ~3 minutes to more than 10 minutes. It is interesting to note that, in the MG1655Δ*cysH* strain, the *csgB* transcript displays an unusual degradation pattern: indeed, after being reduced to ca. 60% within the first two minutes, *csgB* transcript levels seem to remain unaffected until 10 minutes, possibly suggesting the involvement of at least two RNA processing enzymes. On the contrary, *csgD*, *csgF*, and *adrA* mRNA stability is unaffected in the mutant strain (Figure 3C and data not shown), further indication that PAPS accumulation targets specifically *csgBAC* mRNA stability.

Inactivation of *cysH* gene affects composition of outer membrane proteins and cellular adhesion and aggregation. In addition to stimulation of curli fibers production, the *cysH* mutation triggers production of additional cell surface-associated factors, as indicated by the a red phenotype on CR and fluorescent phenotype on CF medium of the MG155Δ*cysH*Δ*csgA* double mutant, observed both at 30 and at 37°C (Figure 1A). In order to identify other extracellular factors influenced by *cysH* inactivation, we carried out protein expression analysis by using Multidimensional Protein Identification Technology (MudPIT) on an outer membrane protein enriched fraction. As the unknown factor(s) is present at 30°C and 37°C in the MG1655Δ*cysH* strain, we considered only the proteins up-regulated in the mutant at the same time at the two temperature (Table 2). Only a really small subset of protein fulfills these criteria. Consistent with increased gene expression levels for curli operons (Figure 3),

CsgA (the main curli subunit), as well as CsgF and CsgG were found to be present at higher levels in the MG1655Δ*cysH* strain. However, curli proteins amount at 37°C are still lower than the one detected at 30°C (data not shown), suggesting that, although synthetized in the Δ*cysH* strain at the higher temperature they might not be enough to stimulate adhesion to solid surfaces.

Beside curli-related proteins, three more proteins were produced at higher levels in MG1655Δ*cysH*, namely: Slp, a lipoprotein induced under carbon starvation or during stationary phase (Alexander and St John, 1994), OmpX, an outer membrane protein with unknown function, but related to virulence (Mecsas et al., 1995; Vogt and Schulz, 1999) and Flu, also known as antigen 43 (Ag43), which can mediate cell-cell aggregation (Danese et al., 2000). No protein involved in exopolysaccharides biosynthesis was found to be up- or down-regulated in the mutant. To investigate whether any of the identified proteins could be responsible of the MG1655Δ*cysH* phenotypes, we inactivated the corresponding genes both in MG1655Δ*cysH* and MG1655Δ*cysH*Δ*csgA* backgrounds. None of the mutations resulted in a reversion to a wild type phenotype in the mutant backgrounds (Figure 4 and data not shown). However, when tested for the adhesion to abiotic surfaces, inactivation of either *slp* or *flu* in the MG1655Δ*cysH* background (Δ*cysH*Δ*slp*, Δ*cysH*Δ*flu*) impaired cell adhesion to polystyrene (Figure 4B). In contrast, similar mutations failed to inhibit cell adhesion in the MG1655, suggesting that Flu and Slp proteins might be required for curli-dependent adhesion in the MG1655Δ*cysH* strain.

Sulphate reduction mutants are not able to metabolize taurine. Our results suggest that PAPS accumulation affects curli production and regulation of the *csgBAC* operon at mRNA stability level (Figure 3, Table 2), and that it can impact EPS production and outer membrane protein composition (Figure 1, Table 2), thus suggesting that PAPS can act as a signal molecule linked to inorganic sulfate availability. Indeed, the sulfate assimilation pathway shown in Figure 2 is only used to synthesize sulfurated amino acids from inorganic sulfate (Kredich, 1992). However, *Escherichia coli* is able to growth in absence of sulfate and cysteine, using aliphatic sulphonates as a source of sulfur (van der Ploeg et al., 2001). The most common alkylsulfonate present in the human bile and large intestine is taurine (Huxtable, 1992): taurine and other aliphatic sulphonates have been proposed to act as signal molecules in several bacteria, such as in the opportunistic pathogen

Burkholderia pseudomallei, where taurine is a strong inducer of flagellar gene expression (Ooi et al., 2013). Although it would be tempting to speculate that PAPS accumulation might partially account for the role of taurine as signal molecule, taurine conversion to cysteine is supposed to be independent of PAPS: indeed, alkane sulphonates are transported into the bacterial cell and converted to sulfite by the products of the *tauABCD* operon. Sulfite is then reduced to sulfide by CysI and then used for cysteine production.

Therefore, we expected that only the MG1655Δ*cysI* strain, which is incapable of reducing sulfite to sulfide, would not be able to grow on a medium with taurine as sole sulphur source. Unexpectedly, however, among all the mutants in the sulfate assimilation pathway, only the MG1655Δ*cysU* strain, impaired in inorganic sulfate uptake, was able to grow (Table 3), indicating that, in our test conditions, taurine utilization requires a fully functional sulphate reduction pathway in the MG1655 strain. Our observations suggest that different sulphur sources, including organosulfonates, might modulate intracellular PAPS concentrations, in turn affecting production of curli fibers and other extracellular structures.

Discussion

In this work, we have shown that the mutation of the *cysH* gene affects the production of different extracellular structures in *E. coli* (Figures 1- 2, Figure 4 and Table 2). These effects are specific to the MG1655Δ*cysH* strain, as no other mutants in the sulphate reduction pathway showed similar phenotypes, and are connected with the accumulation of the PAPS molecule, the substrate of CysH enzyme (Figure 2). We propose that the modified nucleotide phosphoadenosine 5'-phosphosulfate could work as a signal molecule mediating the response to sulphate environmental availability. Several other modified nucleotides act as signalling molecules: cAMP, (p)ppGpp, c-di-GMP regulate different aspects of bacterial life including carbon source utilization, transcription and translation, virulence and biofilm formation (see Kalia et al., 2013 for a complete review). These molecules are synthesized starting from nucleotides through the activity of specific enzymes and are used only to stimulate a cellular response in accordance to environmental and physiological cues they convey. On the contrary, PAPS shows a dual nature being an intermediate of a biochemical pathway required for cysteine biosynthesis and working as a signal molecule. Interestingly,

also adenosine 5'-phosphosulfate (APS), another intermediate of the sulphate reduction pathway, was shown to work as a signal molecule regulating the switch from inorganic to organic sulphur utilization (Bykowski et al., 2002). Although both sulphate intermediates work as signalling molecules they target different processes, and while PAPS seems to regulate a broad spectrum of extracellular structures, APS is more specific in modulating sulphate utilization.

In *E. coli,* we identified curli fibres as the main target of PAPS accumulation. Although curli fibres belong to the CsgD-regulon and are transcriptionally controlled by the CsgD transcriptional regulator, PAPS levels only affect curli acting at *csgBAC* levels as other CsgD-dependent genes, such as *adrA,* were not influenced by the mutation (Figure 3). In addition, either directly or indirectly, the *cysH* mutation affects the production of other surface structures, including the starvation lipoprotein Slp, the outer membrane protein OmpX, the Antigen 43 autotransporter protein and a yet unknown factor of polysaccharidic nature as suggested by CF phenotypes of the MG1655Δ*cysH* strain (Figure 1). Therefore PAPS accumulation not only promotes curli production, but it also results in the reshaping of the cell surface, suggesting a global role for the signalling molecule. Although we were not able to identify the molecular mechanism behind PAPS-dependent regulation, we connected the molecule levels with the stability of the *csgBAC* transcript. Likewise, other modified nucleotides are known cofactors of enzymes involved in RNA metabolism: for instance c-di-GMP and ppGpp were shown to directly regulate the activity of PNPase in *E. coli* and in actinomycetes respectively (Tuckerman et al., 2011; Siculella et al., 2010), while *E. coli* oligoribonuclease (Orn) is inhibited by pAp, one product and direct inhibitor of the CysH catalysed reaction (Mechold et al., 2006). However, we have excluded that the effects of the *cysH* mutation could depend on the pAp molecule as a mutant that accumulates pAp showed similar phenotypes to the *cysH* mutation that are exclusively the result of pAp-dependent inhibition of CysH enzyme (data not shown). Thus, we speculate that like other modified nucleotides, PAPS can directly inhibit one or more enzymes involved in RNA degradation and regulate different processes acting on mRNA stability.

PAPS might be considered another signal for the transition from the nutrient-rich host to the external environment. Shift from a cysteine-rich milieu to a natural environment, where the sulphur amino acids are scarce and inorganic sulphate or alkene-sulfonates are the main sulphur sources, would result in a temporary PAPS

surge and therefore in the activation of PAPS-dependent regulation. Consistent with this observation PAPS accumulation activates production of curli fibres, extracellular structures associated with biofilm formation that are commonly expressed when the bacteria face conditions different from the host (low temperature, low osmolarity) (Römling et al., 1998). Furthermore, we showed that utilization as the sole sulphur source of the organosulfates taurine, the most common alkylsulfonate present in the bile and large intestine (Huxtable, 1992), relay on the presence of an active sulphate reduction pathway (Table 3). A similar result was already seen with MOPS, another organic sulfur source (Baba et al., 2006). Therefore it is tempting to speculate whether PAPS-dependent regulation could be influenced by inorganic and organic sulfur sources both in the host and in the natural environment. Interestingly, the presence of bile, which contains high concentrations of taurine, has been often associated with the induction of extracellular adhesive structures and biofilm formation in many different bacteria (Hung et al., 2006; Ambalam et al., 2012; Pumbwe et al., 2007; Begley et al., 2009). It would be of great interest to establish whether bile-mediate production of biofilm determinants could depend on taurine or the sulfate reduction pathway and in particular on the PAPS molecule.

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Table 1. *Escherichia coli* strains and plasmid used in this work

Strains	Relevant Genotype	Source or Reference
MG1655	K-12, F^- λ^- rph-1	Blattner et al., 1997
MG1655*cysH::Tn5Kan*	Tn5::kan transposon inserted at nucleotide 472 of the *cysH* gene CDS	This work
MG1655Δ*cysH::cat*	Replacement of the *cysH* gene with a chloramphenicol resistance cassette	This work
MG1655Δ*cysH::cat* Δ*csgA::cat*	Obtained by replacing the *csgA* gene with a chloramphenicol resistance cassette in the MG1655Δ*cysH::cat* strain	This work
MG1655Δ*cysH::cat* Δ*bcsA::tet*	Obtained by replacing the *bcsA* gene with a tetracycline resistance cassette in the MG1655Δ*cysH::cat* strain strain	This work
MG1655Δ*cysH::cat* Δ*wcaD::tet*	Obtained by replacing the *wcaD* gene with a tetracycline resistance cassette in the MG1655Δ*cysH::cat* strain strain	This work
MG1655Δ*cysH::cat* Δ*wzyE::tet*	Obtained by replacing the *wzyE* gene with a tetracycline resistance cassette in the MG1655Δ*cysH::cat* strain strain	This work
MG1655Δ*cysH::cat* Δ*pgaA::tet*	Obtained by replacing the *pgaA* gene with a tetracycline resistance cassette in the MG1655Δ*cysH::cat* strain strain	This work
MG1655Δ*cysU::cat*	Replacement of the *cysU* gene with a chloramphenicol resistance cassette	This work
MG1655Δ*cysD::cat*	Replacement of the *cysD* gene with a chloramphenicol resistance cassette	This work
MG1655Δ*cysC::cat*	Replacement of the *cysC* gene with a chloramphenicol resistance cassette	This work
MG1655Δ*cysI::cat*	Replacement of the *cysI* gene with a	This work

	chloramphenicol resistance cassette	
EB1.3	MG1655 *rpoS::tet*	Prigent-Combaret et al., 2001
Plasmids		
pT7-7	Ampicillin resistance, T7 RNA polymerase-dependent promoter	Gualdi et al., 2007
pT7*cysH*$_{wt}$	*cysH* gene cloned into pT7-7 vector as a 735 bp NdeI/PstI fragment	This work
pT7cysH$_{mut}$	*cysH* allele carrying the mutation resulting in the ECGLH → EAGLA change in the CysH redox site	This work

PROTEINS UPREGULATED AT 30°C AND 37°C				
Protein ID	**Protein name**	**DAVE**		**Notes**
		30°C	**37°C**	
Curli encoding proteins				
NP_415560	CsgA	40	160	curlin subunit
NP_415555	CsgG	54	133	curli production assembly/transport outer membrane lipoprotein
NP_415556	CsgF	60	139	outer membrane protein required for curli nucleation by CsgB
Other outer membrane proteins				
NP_417963	Slp	44	77	outer membrane lipoprotein
NP_415335	OmpX	40	35	outer membrane protein X
YP_026164	Flu	200	200	antigen 43 (Ag43) phase-variable biofilm formation autotransporter

Table 2 Protein up-regulated simultaneously at 30°C and 37°C in the outer membrane protein enriched fraction of the MG1655Δ*cysH* strain compared to the corresponding fraction of the MG1655 wild type strain. DAVE=Difference in Average (amount); calculated as in Experimental procedures.

Strain	**OD_{600} (after 48 h of growth)**			
	M9 Glu		SF-M9	
	⦸	0.25 mM tau	⦸	0.25 mM tau
MG1655	1.65	1.43	0.00	0.92
MG1655 Δ*cysU*	0.00	0.63	0.00	0.53
MG1655 Δ*cysD*	0.00	0.00	0.00	0.00
MG1655 Δ*cysC*	0.00	0.00	0.00	0.00
MG1655 Δ*cysH*	0.00	0.00	0.00	0.00
MG1655 Δ*cysI*	0.00	0.00	0.00	0.00

Table 3. Growth was measured as OD_{600} on stationary phase cultures grown for 48 hours at 37°C with vigorous shaking (> 100 rpm) in either M9 Glu or sulphate-free M9 (SF-M9) Glu media in presence or absence (x) of taurine at a final concentration of 0.25 mM.

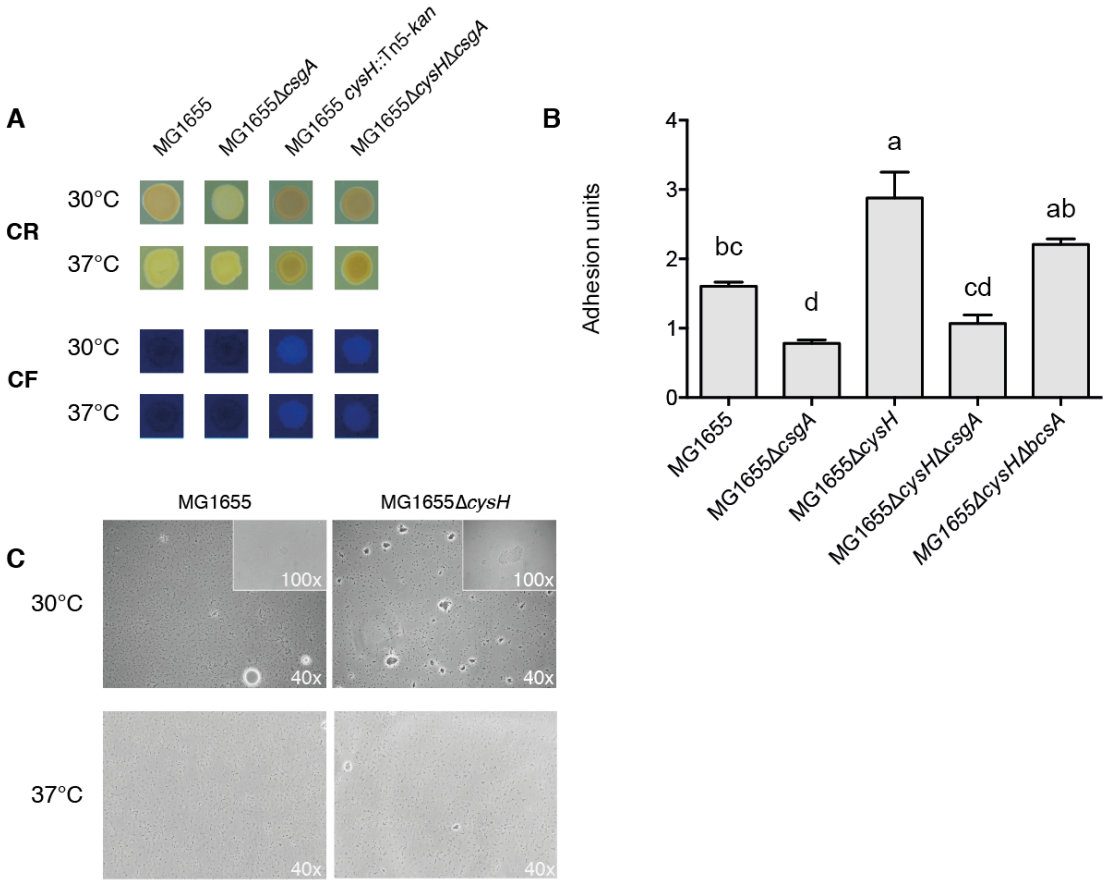

Figure 1. Effects of the *cysH* mutation on CR and CF phenotypes, microtiter adhesion and cellular aggregation. **A.** Phenotypes on CR and CF media of MG1655 (wild type strain), MG1655Δ*csgA* (unable to produce curli), MG1655*cysH*::Tn5-*kan*, MG1655Δ*cysH*Δ*csgA*. Strains were grown either at 30°C (for 24 hours) or at 37°C (for 18 hours). Plates were incubated for 48 hours at 4°C to enhance Congo red and Calcofluor binding. **B.** Surface adhesion of the same set of strains to polystyrene microtiter plates. The adhesion unit values, assessed as previously described (Gualdi et al., 2008), are the average of at least four independent experiments and standard error is shown. Letters provide the representation for posthoc comparisons. According to posthoc analysis (Tukey's HSD, $p < 0.05$), means sharing the same letter are not significantly different from each other. **C.** Phase contrast micrographs (1,000 x magnification) of MG1655, MG1655Δ*cysH*, MG1655Δ*csgA*, MG1655Δ*cysH*Δ*csgA* strains grown overnight in CRL medium at both 30°C and 37°C. The images were acquired with a digital CCD Leica DFC camera.

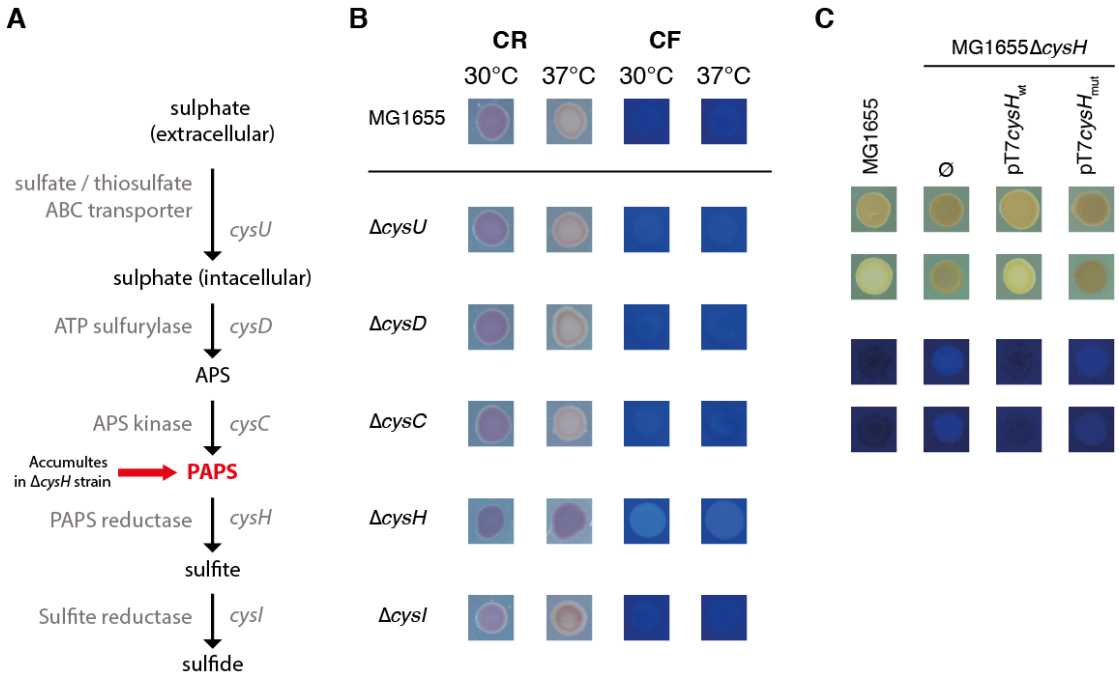

Figure 2. MG1655Δ*cysH* phenotypes depend on PAPS reductase activity and PAPS accumulation. A. Sulphate reduction pathway in *Escherichia coli*. The modified nucleotide phosphoadenosine 5'-phosphosulphate (PAPS) is highlighted in red **B.** Congo red (CR) and Calcofluor (CF) binding by *E. coli* strains deficient in sulphate reduction pathway. MG1655 and isogenic mutants in *cysU*, *cysD*, *cysC*, *cysH*, and *cysI* genes were spotted on both media and grown either at 30°C (for 24h) or at 37°C (18h). Plates were incubated at 4°C for at least 48h to enhance dyes binding. **C.** Effects of *cysH* wild type (pT7cysH$_{wt}$)and mutant (pT7*cysH*$_{mut}$) alleles on MG1655Δ*cysH* CR and CF phenotypes.

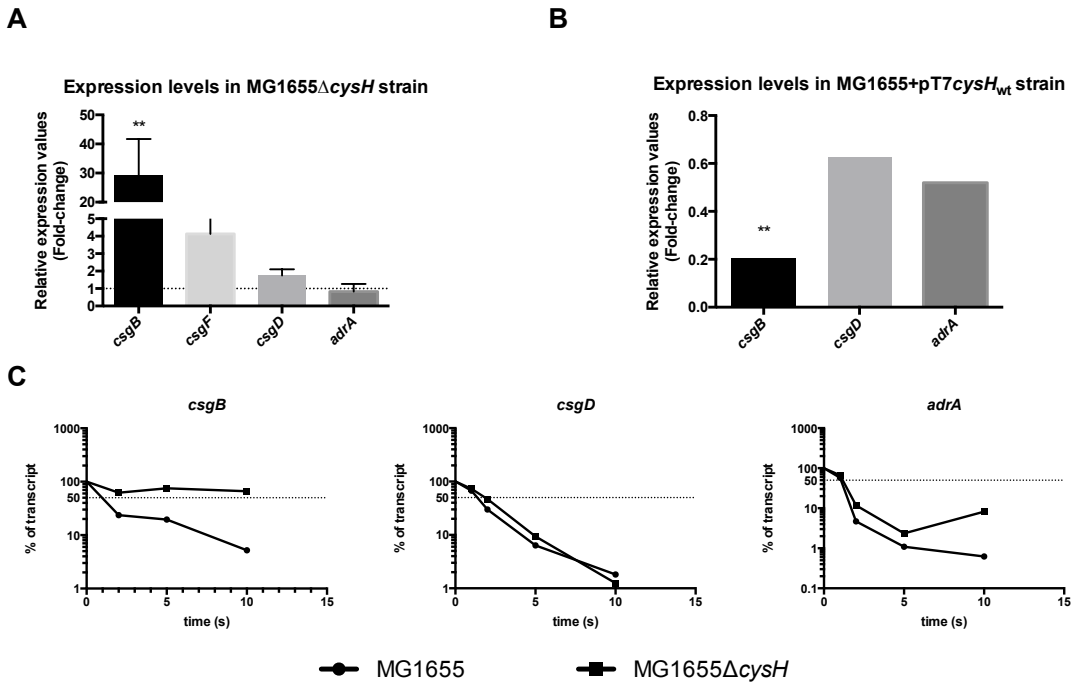

Figure 3. Expression levels of *csgB*, *csgD*, and *adrA* genes in MG1655Δ*cysH* (**A)** and MG1655+pT7*cysH* strains (**B)** measured through qRT-PCR on overnight cultures (~18h) grown at 30°C in CRL medium. ΔCt values between the genes of interest and 16S RNA were set at 1 for MG1655 and MG1655+pT7 (for Panel A and Panel B, respectively), to which expression levels in MG1655Δ*cysH* (Panel A) and MG1655+pT7$cysH_{wt}$ (Panel B) were compared. The reported values are the average of at least four experiments performed on four biological replicates. The asterisks denote significant differences relative to reference strain (** $p < 0.05$; Tukey multigroup analysis) **C.** *csgB*, *csgD*, and *adrA* mRNA decay analysis. MG1655 and MG1655Δ*cysH* cultures were grown overnight at 30°C in CRL medium, rifampicin was added (100 µg/ml) and samples for RNA extraction were taken before (T=0) and after antibiotic addition. Transcript levels of *csgB*, *csgD*, and *adrA* were assayed through qRT-PCR, and mRNA amount at T=0 was set as 100%. Dashed line represent 50% of residual mRNA after rifampicin addition.

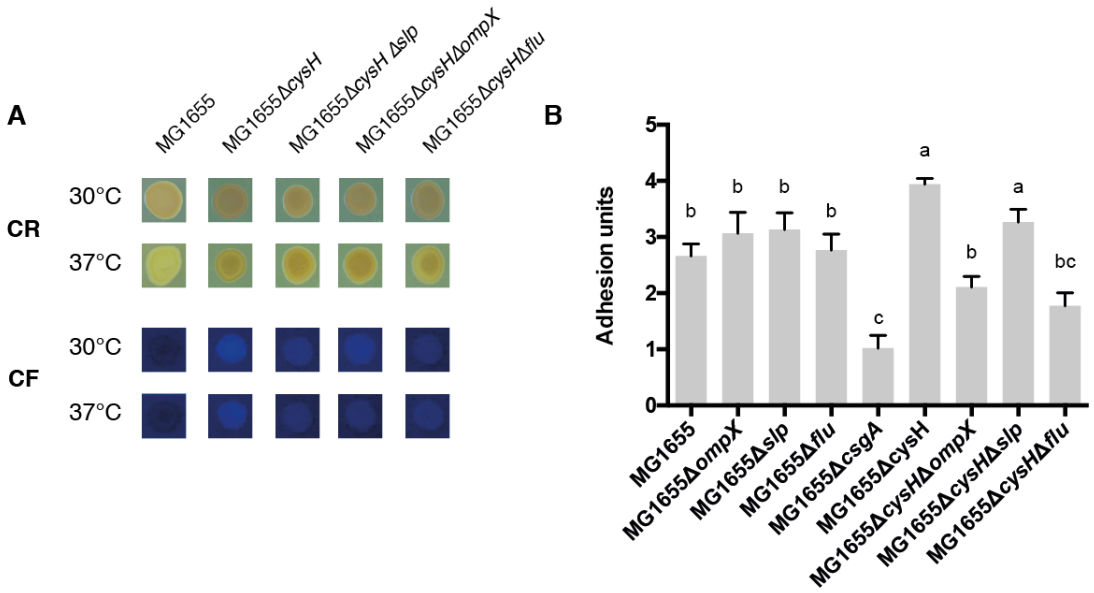

Figure 4. A. Effects of *slp*, *ompX*, and *flu* mutation in the MG1655Δ*cysH* background on Congo red (CR) and Calcofluor (CF) at 30°C and 37°C. **B.** Surface adhesion of *slp*, *ompX*, and flu mutants to polystyrene microtiter plates. The adhesion unit values, assessed as previously described (Gualdi et al., 2008), are the average of at least four independent experiments and standard error is shown. Letters provide the representation for posthoc comparisons. According to posthoc analysis (Tukey's HSD, $p < 0.05$), means sharing the same letter are not significantly different from each other.

Supplementary Figures and Tables

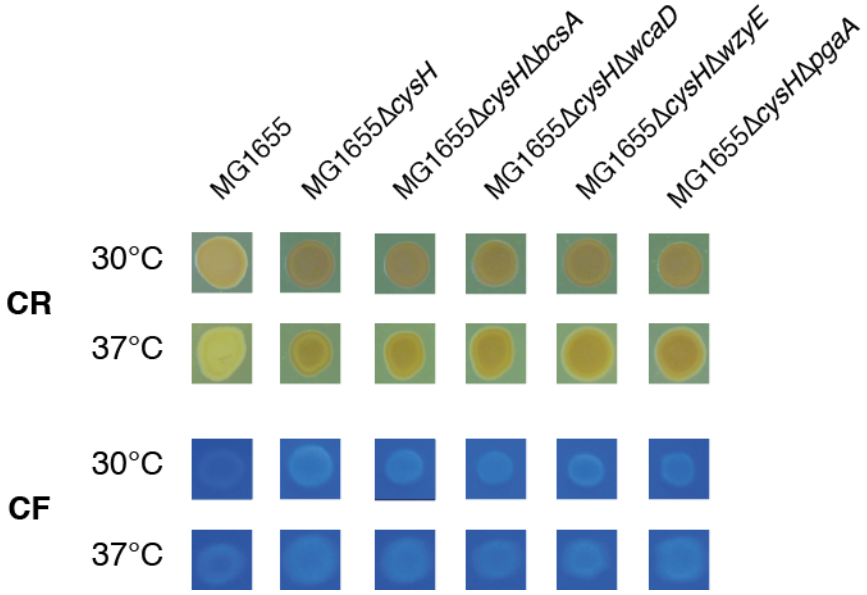

Figure S1. Effects of the inactivation of genes involved in production of *E. coli* most common exopolysaccharides on MG1655Δ*cysH* strain. MG1655, MG1655Δ*cysH,* Δ*cysH*Δ*bcsA* (cellulose), Δ*cysH*Δ*wcaD* (colanic acid), Δ*cysH*Δ*wzyE* (entero common antigen) and Δ*cysH*Δ*pgaA* (PNAG) strains were spotted on CR and CF media and grown either at 30°C (24h) or 37°C (18h). Plates were incubated at 4°C for at least 48h to enhance dyes binding.

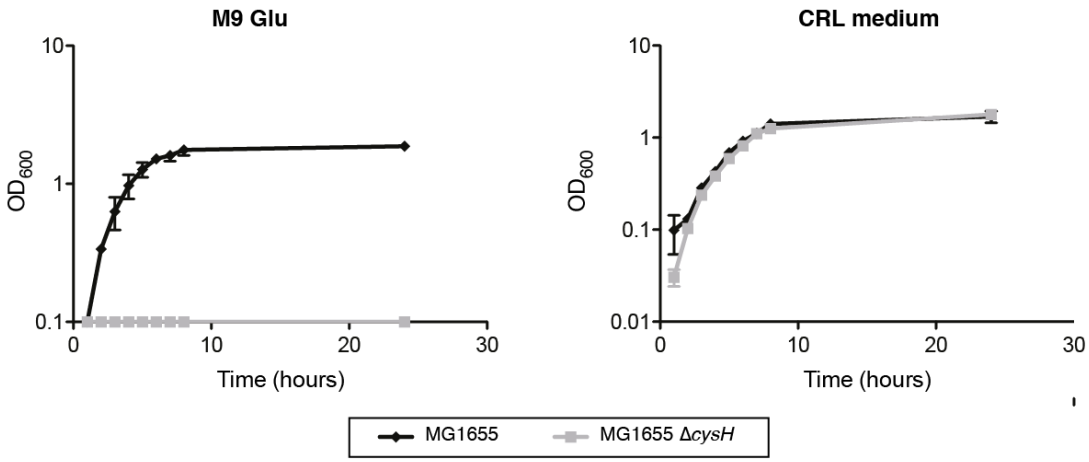

Figure S2. Growth curves of MG1655 (black) and MG1655Δ*cysH* (light gray) in M9 Glu (left panel) and CRL (right panel) media at 37°C. Results are the average of at least three replicates and standard deviation is shown when significant.

Table S1. List of primers used in this work.

Primers	Sequence	Utilization
cysH_NdeI_for	5'-TTCATATGTCCAAACTCGATCTAAACG-3'	*cysH* cloning
cysH_PstI_rev	5'-TTCTGCAGTTACCCTTCGTGTAACCCAC-3'	cysH cloning
cysH_mut_PstI_rev	5'-ttCTGCAGTTACCCTTC**G**GCTAACCC**A**GCTTCCCT-3'	$cysH_{mut}$ cloning
cysC_cat_for	5'-CGCACTGGGGCGCGCGATTTGCTGGGGGATAAATAATGTACCTGTGACGGAAGATCAC -3'	*cysC* inactivation
cysC_cat_rev	5'-ATGGAAACCCGGTGGTGTCTCAGGATCTGATAATATCGTTGGGCACCAATAACTGCCTTA-3'	*cysC* inactivation
cysD_FRT_for	5'-GATGGCAAGAAAATAGCGGTATTGCAAAGGAACGGTTATGTTCCGGGGATCCGTCGACCT-3'	*cysD* inactivation
cysD_FRT_rev	5'-TGCAAGTGCGGTGTTCATCTTAAAAATACCCCTGACGTTTGTGTAGGCTGGAGCTGCTTC-3'	*cysD* inactivation
cysH_FRT_for	5'-GAGCGTCGCATCAGGCAAGGCAAACAGTGAGGAATCTATGGTGTAGGCTGGAGCTGCTTC-3'	*cysH* inactivation
cysH_FRT_rev	5'-TTAATTTGTCCGGCAATATTTACCCTTCGTGTAACCCACATTCCGGGGATCCGTCGACCT-3'	*cysH* inactivation
cysI_cat_for	5'-	*cysI* inactivation

	ATGAGCGAAAAACATCCAGGGCCTTTAGT GGTCGAAGGAATACCTGTGACGGAAGATC AC-3’	
cysI_cat_rev	5’- TTAATCCCACAAATCACGCGCCGGATCGAG CACCGGGCGAGGGCACCAATAACTGCCTT A-3’	*cysI* inactivation
cysU_FRT_for	5’- GCGAGTTAGACAAGCTGTTAGCGGCGGGG CGTAACTGATGGTGTAGGCTGGAGCTGCTT C-3’	*cysU* inactivation
cysU_FRT_rev	5’- CGCTTCAATTGGGTAACTTCCGCCATTAAT GACCTACCACTTCCGGGGATCCGTCGACCT -3’	*cysU* inactivation
bcsA_tet_for	5’- TGAACTACTCCGGGCTGAAAACGCCAGTCG GGAGTGCATCCTAGACATCATTAATTCCTA TGAACTACTCCGGGCTGAAAACGCCAGTCG GGAGTGCATCCTAGACATCATTAATTCCTA- 3’	*bcsA* inactivation
bcsA_tet_rev	5’- CTCAAATGACCGAGCTCTTTTTTCTCATCCA GATGCTGTGGAAGCTAAATCTTCTTTATCC TCAAATGACCGAGCTCTTTTTTCTCATCCA GATGCTGTGGAAGCTAAATCTTCTTTATC-3’	*bcsA* inactivation
wcaD_tet_for	5’- AGCCGCGCCGCCTACAGTGGACAACAGAT GCTGGAGGAGTCTAGACATCATTAATTCCT A-3’	*wcaD* inactivation
wcaD_tet_rev	5’- ATGCTAACGGTAACTTTTTCTTAATCGCCTC TTTATTCAGGAAGCTAAATCTTCTTTATCG-	*wcaD* inactivation

	3’	
wzyE_tet_for	5’-ATGAGTCTGCTGCAATTCAGTGGCCTGTTTGTTGTCTGGCGATTTCAGTGCAATTTATCT-3’	*wzyE* inactivation
wzyE_tet_rev	5’-TTATCCTTCAACCTGCGTCCGGAGCGATGATTTTGTACGTTGAATCCGTTAGCGAGGTGC-3’	*wzyE* inactivation
pgaA_tet_for	5’-ATACAGAGAGAGATTTTGGCAATACATGGAGTAATACAGGGAAGCTAAATCTTCTTTATCGATACAGAGAGAGATTTTGGCAATACATGGAGTAATACAGGGAAGCTAAATCTTCTTTATCG-3’	*pgaA* inactivation
pgaA_tet_rev	5’-ATCAGGAGATATTTATTTCCATTACGTAACATATTTATCCCTAGACATCATTAATTCCTAATCAGGAGATATTTATTTCCATTACGTAACATATTTATCCCTAGACATCATTAATTCCTA-3’	*pgaA* inactivation
pgaA_for	5’- TGGACACTCTGCTCATCATTT-3’	Mutant verification
wzyE _for	5’- AATCCGTTCTGGCAGGA-3’	Mutant verification
wcaD_for	5’- GATATTTGGTACCACGCTC-3’	Mutant verification
bcsA_for	5’- CTAAGCAACCAGTAGGTGAATATC-3’	Mutant verification
cysC_for	5’- GCCAGTTAGCCAGGCAACTGC-3’	Mutant verification
cysD_for	5’- GCTTTGCCAAATCGTTATTCC-3’	Mutant verification
cysH_for	5’- CATTCGCCCGGTGCTCGATC-3’	Mutant verification
cysI_for	5’- CATGGCGAAAGACGTTGAGC-3’	Mutant verification
cysU_for	5’- ACCAGCGGCGGCGAGTTAGA-3'	Mutant verification
P2_rev	5’- TTCCGGGGATCCGTCGACCT-3’	Mutant verification
tet_rev	5’- TGCAGGTAAAGCGATCCCACCAC-3’	Mutant verification
kan-2_rev	5’-ACTCTGGCGCATCGGGCTTC-3’	Transposon insertion

		and mutant verification
R6Kγori_for	5'-CCTCTTTCTCCGCACCCGAC-3'	Transposon insertion verification
16s_for	5’-TGTCGTCAGCTCGTGTCGTGA-3’	qRT-PCR
16s_rev	5’-ATCCCCACCTTCCTCCGGT-3’	qRT-PCR
csgD_RT_for	5’-CCCGTACCGCGACATTG-3’	qRT-PCR
csgD_RT_rev	5’-ACGTTCTTGATCCTCCATGGA-3’	qRT-PCR
csgB_RT_for	5’-CATAATTGGTCAAGCTGGGACTAA-3’	qRT-PCR
csgB_RT_rev	5’-GCAACAACCGCCAAAAGTTT-3’	qRT-PCR
csgF_RT_for	5’- CGCATGGTGACCAACGATTATA-3’	qRT-PCR
csgF_RT_rev	5’- TCTGTCACGTTCAACTGCAATTG-3’	qRT-PCR
adrA_RT_for	5’- GGCTGGGTCAGCTACCAG-3’	qRT-PCR
adrA_RT_rev	5’- CGTCGGTTATACACGCCCG-3’	qRT-PCR

Table S2. Strains growth in M9 Glu with or without 0.25 mM cysteine.

Strain	**OD_{600} (after 24 h of growth)**	
	M9 Glu	
	x	0.25 mM cys
MG1655	1.65	1.37
MG1655*cysH::*Tn5-*kan*	0.00	1.32
MG1655 Δ*cysU*	0.00	1.34
MG1655 Δ*cysD*	0.00	1.31
MG1655 Δ*cysC*	0.00	1.30
MG1655 Δ*cysH*	0.00	1.33
MG1655 Δ*cysI*	0.00	1.28
MG1655 Δ*cysH* + pT7-7	0.00	not tested
MG1655 Δ*cysH* + pT7*cysH*$_{wt}$	1.62	not tested
MG1655 Δ*cysH* + pT7*cysH*$_{mut}$	0.00	not tested

Growth was measured as OD_{600} on stationary phase cultures grown for 24 hours at 37°C with vigorous shaking (> 100 rpm) in M9 media in presence or absence (x) of cysteine at a final concentration of 0.25 mM.