Escherichia coli Biofilms

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▶ **To cite this version:**

Christophe Beloin, Agnès Roux, Jean-Marc Ghigo. Escherichia coli Biofilms. Tony Romeo. Bacterial Biofilms, 322, Springer, pp.249-289, 2008, Current Topics in Microbiology and Immunology, 978-3-540-75417-6. 10.1007/978-3-540-75418-3_12 . pasteur-00473297

HAL Id: pasteur-00473297

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Submitted on 15 Apr 2010

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<metadata>
<doi>b11738312-0012
<en>
<articletype>original paper
<producttype>monograph
<subject>life sciences
<maketoc levels="2"/>
<numberingstyle>ContentOnly
<chapternumber>12
</metadata>

<header>
<title>*Escherichia coli* Biofilms
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<abstract>
<formalp><phead1>Abstract</phead1><p>*Escherichia coli* is a predominant species among facultative anaerobic bacteria of the gastrointestinal tract. Both its frequent community lifestyle and the availability of a wide array of genetic tools contributed to establish *E. coli* as a relevant model organism for the study of surface colonization. Several key factors, including different extracellular appendages, are implicated in *E. coli* surface colonization and their expression and activity are finely regulated, both in space and time, to ensure productive events leading to mature biofilm formation. This chapter will present known molecular mechanisms underlying biofilm development in both commensal and pathogenic *E. coli.*
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<heading1>Introduction

<p>The description of a widespread association between bacteria and surfaces dates back to the dawn of microbiology when, in the seventeenth century, Antone Van Leeuwenhoek observed "animalcules" on his own dental plaque surface. Since then, however, what we now know to be bacteria have most often been studied in artificial but controlled conditions using agitated single-celled planktonic cultures. It is currently admitted that we have explored only a very specific aspect of bacterial biology since, in

most ecological niches, bacterial interactions with a surface promote novel behaviors leading to the development of structured and heterogeneous, matrix-encased bacterial communities known as biofilms. Biofilm physiology is characterized by increased tolerance to stress, biocides (including antibiotics) (see the chapter by G.G. Anderson and G.A. O'Toole, this volume) and host immunological defenses, which is at the origin of their resilience in most medical and industrial settings. During the last decade, the negative impact of biofilm development on human activities has stimulated research aimed at providing clues for combatting detrimental biofilms. Most recent studies conducted on genetic requirements underlying bacterial biofilm formation have used a limited selection of model bacteria, including *Escherichia coli*.

<p>Although current knowledge of bacterial biology owes much to work done on planktonic cultures of laboratory strains of *E. coli,* many isolates also have the capacity to form biofilm structures in vivo and in vitro**.** Indeed, *E. coli* is a predominant species among facultative anaerobic bacteria of the gastrointestinal tract, where it thrives in an environment with structural characteristics of a multispecies biofilm (Costerton et al. 1995; Probert and Gibson 2002).

<p>With over 250 serotypes, *E. coli* is a highly versatile bacterium ranging from harmless gut commensal to intra- or extraintestinal pathogens, including common colonizers of medical devices and the primary causes of recurrent urogenital infections (Kaper et al. 2004).

<p>In this chapter dedicated to *E. coli* biofilms, we will attempt to provide an overview of present knowledge of molecular mechanisms underlying surface colonization by *E. coli*. While particular emphasis will be placed on studies conducted on *E. coli* K-12, in vivo aspects of pathogenic and natural *E. coli* isolate biofilm formation will also be presented.

<heading1>First Contacts with the Surface

<heading2>Approaching the Surface: The role of Motility in *E. coli* Adhesion to Surfaces

<p>In a liquid environment, bacteria are subjected to hydrodynamic forces, especially when approaching surfaces. Alongside passive movement that is governed by Brownian or gravitational forces, bacteria have developed mechanisms of active motility that enable them to overcome repulsive electrostatic and hydrodynamic forces encountered around surfaces, and consequently to increase their chances of interacting with surfaces (Donlan 2002).

<p>In Gram-negative bacteria such as *E. coli* and *Salmonella*, active motility is dependent on a flagellar apparatus that is necessary for them to swim in liquid or semi-

liquid medium. In one of the earliest studies using *E. coli* as a model system, half of the insertion mutants deficient at biofilm formation identified by Pratt and Kolter were found to perturb flagellar functions (Pratt and Kolter 1998). These authors showed that motility itself, and not chemotaxis or direct surface contact by flagella, was required to form a biofilm, and they proposed that, beyond allowing the bacteria to breach repulsive forces, flagella may also allow bacteria to spread along the surface. This analysis was confirmed in *E. coli* by Genevaux and co-workers (Genevaux et al. 1996) and, more recently, by Wood and co-workers, who demonstrated, using a set of different motility mutants, that the biofilm formation capacities of *E. coli* K-12 were directly correlated with its ability to swim (Wood et al. 2006).

<p>Although this suggests that the requirement for force-generating cell-surface organelles is a common theme in biofilm formation, it is not an absolute requirement, and nonmotile bacteria can still form biofilms under certain conditions, as shown for *E. coli* K-12 (Pratt and Kolter 1999), but also for enteroaggregative *E. coli* (EAEC) (Sheikh et al. 2001). In a nonmotile strain that overexpresses the surface adhesins known as curli (see below), Prigent-Combaret and co-workers showed that flagellar motility is not required for initial adhesion, nor is it required for biofilm development (Prigent-Combaret et al. 2000). Reisner and co-workers showed that in an *E. coli* strain bearing a conjugative plasmid, known as a strong adhesion factor (Ghigo 2001), the presence of flagellum was dispensable for biofilm formation (Reisner et al. 2003). In these cases, it is possible that the expression of strong adhesion factors may replace force-generating movements during initial interactions between adhering bacteria and the surface (Pratt and Kolter 1999; Geesey 2001; Donlan 2002).

<heading2>Primary Adhesion to Surfaces: Reversible Attachment

<p>Whereas flagellar motility helps the bacteria to counteract hydrodynamic and electrostatic forces near the surface, initial bacterial adhesion to abiotic surfaces is likely to be highly dependent on physicochemical and electrostatic interactions between the bacterial envelope itself and the substrate, which is often conditioned by the fluids to which it is exposed (Dunne 2002). Attracting and repulsing forces between the bacteria and the surface lead to reversible attachment of bacteria to the surface, with most of the bacteria leaving the surface to join the planktonic phase either because of mild shear or the bacterium's own mobility (Dunne 2002). This reversible attachment is strongly influenced both by environmental conditions such as pH and the ionic force of the medium or the temperature (Fletcher 1988; Danese et al. 2000a), and by the nature of

the surface itself, with rugosity increasing surface adhesion or hydrophobic surfaces such as Teflon or plastic more likely to be colonized by bacteria than hydrophilic surfaces such as glass and metal (Donlan 2002). Furthermore, adsorption and desorption of nutrients at the surface, which compose a so-called conditioning film, are also important factors that may influence bacterial initial attachment both positively and negatively depending on the nature of the organic molecules involved (Zobell 1943; van Loosdrecht et al. 1990).

<heading2>Irreversible Adhesion to Surfaces: The Role of Fimbriae

<p>Alongside thermodynamic aspects, the direct contribution of adhesive organelles of the fimbrial family to the irreversible attachment of bacteria to surfaces has been amply demonstrated. Three classes of fimbriae have a role in strengthening the bacteria-to-surface interactions: type 1 fimbriae, curli, and conjugative pili.

<heading3>Type 1 Fimbriae

<p>Type 1 fimbriae (or pili) are filamentous proteinaceous adhesins commonly expressed both by commensal and pathogenic *E. coli* isolates (Sauer et al. 2000). Bacteria expressing type 1 fimbriae generally present between 100 and 500 fimbriae at their surface. These fimbriae have a tubular structure that is 5–7 nm in diameter and between 0.2 and 2 μm long. Type 1 pili can adhere, in a mannose-dependent manner, to a variety of receptor molecules on eukaryotic cell surfaces (Duncan et al. 2005) and are well documented virulence factors in pathogenic *E. coli* (Kaper et al. 2004). Along with their role in the formation of secreted IgA-mediated biofilm within the gut (Bollinger et al. 2003, 2006; Orndorff et al. 2004), several groups have reported that these adhesins are critical for *E. coli* biofilm formation on abiotic surfaces (Harris et al. 1990; Pratt and Kolter 1998; Cookson et al. 2002; Moreira et al. 2003; Orndorff et al. 2004). Mutants in both *fimA,* the gene encoding the major type 1 pilus subunit, and *fimH*, which codes for the mannose-specific adhesin located at the tip of the pilus, have been reported to reduce *E. coli* initial attachment to polyvinyl chloride and other abiotic surfaces (Pratt and Kolter 1998; Beloin et al. 2004). These findings suggest that the type 1 pili FimH adhesin, besides binding eukaryotic mannose oligosaccharides, may also have nonspecific binding activity at abiotic surfaces (Pratt and Kolter 1998). The expression of type 1 pili is induced by adhesion and biofilm formation at early and late stages (Schembri et al. 2003b; Beloin et al. 2004; Ren et al. 2004a).

<p>While the FimH adhesin itself could be responsible for this nonspecific binding, several studies have indicated that the expression of type 1 pili may affect adhesion of *E.*

coli to abiotic surfaces by altering the composition of the outer membrane (Orndorff et al. 2004). Indeed, Otto and co-workers showed that type 1 pili surface contacts mediate a decrease in the abundance of several outer membrane proteins such as BtuB, EF-Tu, OmpA, OmpX, Slp, and TolC (Otto et al. 2001). These changes in the envelope probably affect the general physicochemical characteristics of the bacterial surface and thereby influence adhesion (Otto et al. 2001). Consistently, production of type 1 pili is upregulated in the absence of OmpX. Furthermore, the absence of OmpX also increases exopolysaccharide production as well as decreasing motility of the bacteria. The decrease in the OmpX level upon type 1 pili-mediated surface contacts may therefore serve as a signal leading to physiological adaptation in surface-associated bacteria (Otto and Hermansson 2004).

<p>A recent work by Lacqua and collaborators showed that when *E coli* K-12 MG1655 is mixed with P1*vir* or λ phages, a phage-tolerant population with increased biofilm formation capacities arose within 24 h, and this appearance was dependent on the presence of type 1 fimbriae. This suggested that type 1 fimbriae-mediated abiotic biofilm formation by K-12 *E. coli* MG1655strain might represent a strategy to escape bacteriophage attack, thus emphasizing the importance of type 1 fimbriae in *E. coli* physiology (Lacqua et al. 2006).

<heading3>Curli Fimbriae

<p>Initially identified in *E. coli*, curli fimbriae, also called thin aggregative fimbriae, are produced by other *Enterobacteriaceae* such as *Shigella*, *Citrobacter*, and *Enterobacter* (Smyth et al. 1996). Curli fimbriae aggregate at the cell surface to form 6- to 12-nm-diameter structures whose length varies between 0.5 and 1 μm. Curli have been demonstrated to attach to proteins of the extracellular matrix such as fibronectin, laminin, and plasminogen (Olsen et al. 1989; Ben Nasr et al. 1996), thus promoting adhesion of the bacteria to different human cells. In addition, curli adhesive fibers also promote biofilm formation to abiotic surfaces both by facilitating initial cell–surface interactions and subsequent cell–cell interactions (Vidal et al. 1998; Cookson et al. 2002; Uhlich et al. 2006). Genes involved in curli production are clustered in two divergently transcribed operons: the *csgBA* operon, encoding the structural components of curli, the *csgDEFG* operon, encoding a transcriptional regulator (CsgD) and the curli export machinery (CsgE-G). In environmental and clinical isolates of *E. coli*, the synthesis of curli is subject to tight and complex regulation allowing curli production notably at 37°C and/or 28°C depending on the isolates, whereas the expression of curli is cryptic in most *E. coli*

laboratory strains. However, Vidal and co-workers isolated, in this type of domesticated strain, a gain-of-function mutant with increased capacity for surface adhesion and cell-to-cell interactions due to the constitutive expression of the cell-surface adhesin curli (Vidal et al. 1998). They determined that the hyperadhesive phenotype was the result of a point mutation in the OmpR protein that constitutes, with the EnvZ protein, a two-component regulatory system that senses variations in osmolarity. This *ompR* allele (*ompR*234) leads to more efficient OmpR-dependent activation of the *csgD* promoter, which stimulates curli production and biofilm formation in laboratory strains (Vidal et al. 1998; Prigent-Combaret et al. 2000; Prigent-Combaret et al. 2001).

<p>Along with the EnvZ/OmpR two-component system, several transcriptional regulators (CpxR, RcsCDB, RpoS, H-NS, IHF, Crl, MlrA) responding to different environmental and stress conditions such as temperature, osmolarity, pH and oxygenation are involved in regulation of curli expression through a network of interactions between transcription factors and the *csg* regulatory region (Dorel et al. 1999; Prigent-Combaret et al. 2001; Brombacher et al. 2003; Gerstel et al. 2003; Jubelin et al. 2005; Vianney et al. 2005). This complex regulatory network is presumed to allow fine-tuning of curli expression that may play a role in colonization of specific niches by *E. coli,* especially in the human body (Prigent-Combaret et al. 2001; Kikuchi et al. 2005).

<heading3>Conjugative Pili

<p>Although most laboratory *E. coli* K-12 strains are poor biofilm formers, the introduction, either artificially or naturally, in mixed *E. coli* communities of a conjugative plasmid in these strains induces formation of a thick mature biofilm (Ghigo 2001; Reisner et al. 2003, 2006). Mutational analysis of the conjugative transfer apparatus genes of the F plasmid demonstrated that this phenotype does not depend on the ability of the plasmids to mediate DNA transfer, but it does require a functional conjugative pilus (Ghigo 2001). The F-pilus promotes both initial adhesion and biofilm maturation through nonspecific attachment to abiotic surfaces and subsequent cell-to-cell contacts, which stabilize the structure of the biofilm (Ghigo 2001; Molin and Tolker-Nielsen 2003; Reisner et al. 2003). Reisner and co-workers also showed that expression of the F conjugative pilus could functionally substitute for other known adhesion factors such as type1 pili, Ag43 or curli (Reisner et al. 2003). Plasmid-mediated biofilm production is not restricted to the F plasmids, and most tested conjugative plasmids directly contribute, upon derepression of their conjugative function, to bacterial host capacity to form a biofilm (Ghigo 2001). This general connection between conjugation and biofilm formation is

consistent with early observations showing that surface contacts positively affect the dynamics of plasmid transfer (Simonsen 1990). Since then, numerous studies investigating transfer of both conjugative and nonconjugative plasmids indicate that physical contact between donor and recipient cells is highly favored within monospecific and mixed *E. coli* biofilms, where efficient horizontal transfer of genetic material has been demonstrated (Lebaron et al. 1997; Hausner and Wuertz 1999; Licht et al. 1999; Dionisio et al. 2002; Molin and Tolker-Nielsen 2003; Maeda et al. 2006). In addition, those studies showed that both conjugative and nonconjugative plasmids are likely to carry determinants for biofilm initiation and architecture, thus promoting biofilm development, which in turn affects the extent of plasmid-mediated horizontal gene transfer within the biofilm (Wuertz et al. 2004).

<p>Several cell adhesins encoded by genes on plasmids or mobile genetic elements have been characterized in pathogenic *E. coli* (Henderson et al. 1998; Kaper et al. 2004; Dudley et al. 2006), suggesting that the extrachromosomal gene pool (plasmids and other mobile genetic elements) may also constitute an important source of adhesion factors leading to biofilm formation, influencing both the probability of biofilm-related infection and of conjugational spread of plasmid-borne virulence factors (Amabile-Cuevas and Chicurel 1996; Ghigo 2001; Molin and Tolker-Nielsen 2003).

<p>This section has presented both environmental and bacterial structures leading to irreversible surface attachment. The section that follows will address the question of biofilm maturation, whereby interbacterial adhesion, rather than direct contact with the substrate, leads to progressive buildup of the mature biofilm.

<heading1>Building the Mature Biofilm

<p>Biofilm maturation corresponds to the three-dimensional growth of the biofilm that occurs after initial attachment to the surface. Mostly due to bacterium–bacterium interactions, this process leads to the formation of a heterogeneous physicochemical environment in which biofilm bacteria display characteristic physiological traits that distinguish them from their planktonic counterparts. Whereas the delimitation between initial attachment and maturation is gradual – both processes involve some of the structures described in the previous section – we will now describe the surface proteins and extracellular matrix components particularly involved in bacterial interadhesion and biofilm architecture.

<heading2>Surface Adhesins that Contribute to Biofilm Structure

<heading3>Autotransporter Adhesins

<p>In most Gram-negative bacteria, the translocation of proteins toward the extracellular medium requires crossing of the cytoplasmic membrane, the periplasm, and the outer membrane. This translocation can be achieved through at least six different secretion pathways (Economou et al. 2006). Among them, the type V secretion pathway enables a family of proteins to reach the surface with a very limited number of accessory secretion factors because most information necessary to the translocation process is contained within the secreted protein itself. These proteins, which can therefore carry out their own transport to the outer membrane, are called autotransported or autotransporter proteins.

<heading4>The Type V Secretion Pathway

<p>Pohlner and collaborators were the first to describe a model of translocation of the *Neisseria gonorrhoeae* IgA1 protease by the type V secretion pathway (Pohlner et al. 1987). Since then, many autotransporter proteins have been described. Each of these proteins displays a modular structure with four characteristic domains: (1) an N-terminal signal peptide that allows translocation through the cytoplasmic membrane via the Sec general pathway; (2) a passenger alpha domain that provides functionality to the secreted proteins and is exposed to the cell surface or released in the extracellular medium; (3) a linker necessary for translocation of the passenger domain through the outer membrane; and (4) a C-terminal beta-domain forming a transmembrane pore (Henderson et al. 2004). The study of the mechanisms of type V secretion showed that, once exported through the cytoplasmic membrane via the mechanism of Sec secretion, the signal peptide is then cleaved by a peptidase that releases a mature protein into the periplasm. The beta-domain of the now periplasmic protein is then thought to insert spontaneously and form a transmembrane beta-barrel pore, enabling translocation of the passenger domain onto the surface of the cell. Though this was initially thought to be an autonomous process, some accessory factors such as the Omp85 protein are nevertheless involved in insertion of autotransporter proteins into the outer membrane (Voulhoux et al. 2003; Oomen et al. 2004). However, Omp85 also seems to be required for other outer membrane protein insertions and is therefore not specific to the type V secretion pathway. The passenger domain can then either undergo self autocatalytic cleavage or remain attached to the external membrane (Henderson et al. 1998). Autotransporter proteins were identified in most Gram-negative bacteria and were classified into three families according to the function carried by their passenger alpha domain: proteases such as the IgA1 protein, esterases, such as ApeE of *Salmonella typhimurium* and adhesins

(Henderson et al. 1998).

<heading4>Antigen 43

<p>In 1980, B. Diderichsen identified an *E. coli* gene whose mutation affects various phenotypes associated with the surface properties of the bacteria. He showed that a change in this gene, called *flu*, prevented flocculation of bacteria at the bottom of a tube and modified the morphology of colonies on agar plates (Diderichsen 1980). The *flu* gene encodes antigen 43 (Ag43), a major outer membrane protein found in most commensal and pathogenic *E. coli*. Although *E. coli* K-12 has only one copy of *flu*, most other strains of *E. coli* have several copies of this gene.

<p>Ag43 is a self-recognizing surface autotransporter protein that does not seem to be involved in non-specific initial adhesion to abiotic surfaces, but rather, promotes cell-to-cell adhesion (Kjaergaard et al. 2000a). While, in liquid culture, this property leads to autoaggregation and clump formation rapidly followed by bacterial sedimentation, it also facilitates bacteria–bacteria adhesion and leads to the three-dimensional development of the biofilm (Owen et al. 1996; Henderson et al. 1997a; Hasman et al. 1999; Kjaergaard et al. 2000a; Schembri et al. 2003a). When expressed in different species, Ag43 can also be used to promote mixed biofilm formation between different bacteria, for example, between *E. coli* and *Pseudomonas aeruginosa* (Kjaergaard et al. 2000a, 2000b).

<p>Hence, Ag43 seems to play a key role in biofilm maturation on abiotic surfaces, but also in eukaryotic cells, where a recently discovered glycosylation reaction on Ag43 could play a role, suggesting a link between expression of Ag43 and the ability of pathogenic *E. coli* to adhere to and form biofilm-like structures on epithelial cells (see below) (Anderson et al. 2003; Justice et al. 2004; Sherlock et al. 2006). Several groups have consistently demonstrated that there is a correlation between an increased level of *flu* expression and *E. coli* capacity to form a biofilm on different abiotic surfaces (Danese et al. 2000a; Kjaergaard et al. 2000a, 2000b; Beloin et al. 2006). Moreover, global gene expression studies showed that the biofilm lifestyle is often associated with increased expression of *flu* as compared to planktonic growth (Schembri et al. 2003b).

<heading4>AidA and TibA Proteins

<p>AidA adhesin from diarrhea-causing *E. coli* and TibA adhesin/invasin associated with some enterotoxigenic *E. coli* are two glycosylated surface proteins involved in bacterial adhesion to a variety of eukaryotic cells. Both AidA and TibA are autotransporter proteins sharing approximately 25% identity at the sequence level with Ag43, and they play a role

in the virulence of different pathogenic *E. coli* strains. Recent studies have shown that, in addition to autoaggregation, expression of these proteins also promotes biofilm formation on abiotic surfaces (Sherlock et al. 2004, 2005). Though they are quite different with respect to size, glycosylation, and processing, these three proteins share common properties: all are self-associating proteins that cause bacterial aggregation and enhance biofilm formation. They can also interact with each other via heterologous interactions, promoting the formation of mixed bacterial aggregates. Based on these properties, Klemm and co-workers proposed that they be classified together in a subgroup termed SAAT, for self-associating autotransporters (Klemm et al. 2006).

<heading3>Exploring the Adhesin Potential of *E. coli*

<p>Genetic analyses have revealed the diversity of *E. coli* adhesins contributing either to colonization or biofilm maturation. Few, if any, of these adhesion factors are absolutely required for biofilm formation; instead, they can be replaced by alternative adhesion factors. A recent study demonstrated that four previously uncharacterized *E. coli* genes (*yfaL*, *yeeJ*, *ypjA*, and *ycgV)*, sharing homologies with the autotransporter adhesin Ag43 and biofilm-associated proteins (Bap) (Cucarella et al. 2001; Latasa et al. 2005), lead to clear adhesion and a biofilm phenotype when expressed from a chromosomally introduced inducible promoter. Deletion of genes coding for these putative adhesins does not significantly alter the adhesion phenotype of the wild type MG1655 *E. coli* K-12 strain under laboratory conditions, indicating that these genes may be cryptic (Roux et al. 2005).

<p>Those studies demonstrated that *E. coli* K-12 probably possesses a large and partly unexplored arsenal of surface adhesins with different binding specificities that are expressed under specific physiological conditions, possibly in response to different environmental cues. This adhesion potential is likely to be even greater in some pathogenic *E. coli* isolates, which not only often have a larger genome than *E. coli* K-12 and therefore express new types of adhesin or fimbrial structures (Buckles et al. 2004), but also carry several plasmids that can contribute to adherence to cells and abiotic surfaces (Perna et al. 2001; Dobrindt et al. 2002; Welch et al. 2002; Dudley et al. 2006).

<heading2>Biofilm Matrix Polysaccharides

<p>One of the most distinctive features that distinguishes biofilms from planktonic populations is the presence of an extracellular matrix embedding the biofilm bacteria and determining mature biofilm architecture (Sutherland 2001; Starkey et al. 2004). Along with

expression of proteinaceous adhesins, production of this matrix is essential for maturation of the biofilm structure. The biofilm matrix is a complex milieu essentially composed of water (97%), but it also includes exopolysaccharide polymers, proteins, nucleic acids, lipids/phospholipids, absorbed nutrients, and metabolites (Ghannoum and O'Toole 2001).

<heading3>Role of the Biofilm Matrix

<p>Although the matrix is a hallmark of bacterial biofilms, its role is not fully understood. The biofilm matrix offers a constantly hydrated viscous layer protecting embedded bacteria from desiccation or from host defenses by preventing recognition of biofilm bacteria by the immune system. The matrix may also play a significant protective role as a diffusion barrier and a sink for toxic molecules (antimicrobials, hydroxyl radicals, and superoxide anions). The biofilm matrix could also inhibit wash-out of enzymes, nutrients, or even signaling molecules that could then accumulate locally and create more favorable microenvironments within the biofilm (Redfield 2002; Welch et al. 2002; Starkey et al. 2004). All these aspects of the putative roles of the matrix could contribute to development of phenotypic resistance of pathogenic *E. coli* biofilms and lead to persistent infections (Anderson et al. 2003; Justice et al. 2004).

<p>In addition to its protective role, one of the main functions of the matrix is probably also a structural one. The adhesive properties of the matrix enable the bacteria to remain in proximity to the surface and to adhere to each other. Moreover, the interactions between polysaccharides and the other components of the matrix, such as those between cellulose and curli, may participate in three-dimensional growth of the biofilm (White et al. 2003).

<p>Due to biofilm heterogeneity, analysis of the extracellular polymeric substance (EPS) is progressing slowly, and little is yet known about the composition of the biofilm matrix (Sutherland 2001). Several exopolysaccharides found in the *E. coli* biofilm matrix (cellulose, PGA, colanic acid) are key components of the biofilm matrix, while others such are lipopolysaccharides and capsular polysaccharides may not accumulate significantly in the matrix, but still play an important indirect role in biofilm formation. While these components may coexist in the matrix, our current knowledge seems to indicate that they are subject to very distinct regulatory pathways, the coordinated expression of which remains to be clarified.

<heading3>Polysaccharides Secreted in the Biofilm Matrix

<p>Secreted polysaccharides have been recognized as key elements that shape and

provide structural support for the biofilm (Sutherland 2001). These polymers are very diverse and are often involved in the establishment of productive cell-to-cell contacts that contribute to the formation of biofilms at liquid–solid interfaces, pellicles at air–liquid interfaces, cell aggregates and clumps in liquid cultures, and wrinkled colony morphology on agar plates. Evidence for a structural role of some of these matrix polysaccharides is accumulating, and the regulation of production of these exopolysaccharides is now actively being investigated in different bacteria (Kirillina et al. 2004; Branda et al. 2005; Simm et al. 2005). To date, three exopolysaccharides, β-1,6-N-acetyl-D-glucosamine polymer (PGA*)*, colanic acid, and cellulose, have been detected in the biofilm matrix of *E. coli* and have been shown to be important for biofilm formation.

<heading4>Poly-β-1,6-N-acetyl-glucosamine

<p>β-1,6-*N*-acetylglucosamine (β-1,6-GlcNAc) is a polysaccharide polymer known to participate in biofilm formation in *Staphylococcus aureus* and *Staphylococcus epidermidis,* where it contributes to their virulence (Mack et al. 1996; Rupp et al. 2001; Gotz 2002; Maira-Litran et al. 2002). β-1,6-GlcNAc, or PGA, was recently identified in *E. coli* K-12, where the expression of β-1,6-GlcNAc exopolysaccharide polymer is involved in both cell–cell adhesion and attachment to surfaces (Agladze et al. 2005). Moreover, PGA depolymerization by treatment with metaperiodate or a β-hexosaminidase from *Actinobacillus actinomycetemcomitans* (DspB), which degrade the β-1,6-GlcNAc, leads to nearly complete disruption and dispersion of the biofilm (Wang et al. 2004; Itoh et al. 2005). PGA production depends on the *pgaABCD* locus (Wang et al. 2004). The *E. coli pgaABCD* (or *ycdSRQP*) operon encodes proteins involved in the synthesis (the PgaC glycosyltransferase), export and localization of the PGA polymer. The *pgaABCD* operon exhibits features of a horizontally transferred locus and is present in a variety of eubacteria. Therefore, it has been proposed that β-1,6-GlcNAc serves as an adhesin that stabilizes biofilms of *E. coli* and other bacteria such as *Actinobacillus actinomycetemcomitans* and *Actinobacillus pleuropneumoniae* (Kaplan et al. 2004; Wang et al. 2004).

<heading4>Cellulose

<p>Cellulose, the main component of plant cell wall, is a homopolysaccharide composed of D-glucopyranose units linked by β-1→4 glycosidic bonds. Outside of the plant kingdom, cellulose has primarily been thought to be produced only by a few bacterial species such as the model organism *Gluconacetobacter xylinum* (Czaja et al. 2006). The

ability of cellulose to bind fluorescent chemical dyes such as calcofluor has provided a convenient screen for cellulose-producing bacteria, showing that cellulose production is a widespread phenomenon in Enterobacteriaceae, including *Salmonella enterica* serovar Typhimurium, *Salmonella enterica* subsp. *Enterica* serovar Enteritidis, and commensal and pathogenic strains of *E. coli*, *Citrobacter spp.* and *Enterobacter spp.* (Zogaj et al. 2001; Solano et al. 2002; Romling et al. 2003; Zogaj et al. 2003; Romling 2005; Da Re and Ghigo 2006; Uhlich et al. 2006). In these bacteria, cellulose production is clearly associated with the ability to form a rigid biofilm at the air–liquid interface; however, these characteristics vary between strains and serovars and are highly dependent on environmental conditions.

<p>Genetic analysis performed in *Salmonella* serovar Typhimurium and *Salmonella* serovar Enteritidis showed that cellulose synthesis genes are organized as two divergently transcribed operons, *bcsABZC* and *bcsEFG*, which are constitutively expressed and composed of genes sharing homologies with genes of the bacterial cellulose operon of *G. xylinum* (Gerstel et al. 2003; Romling 2005).

<p>Although these genes are present in most enterobacterial genomes, including *Salmonella, E. coli, Shigella, Enterobacter*, and *Citrobacter* (Zogaj et al. 2003), little is known about the function and localization of the bacterial cellulosome. BcsA is a cytoplasmic membrane protein whose cellulose synthase activity is allosterically controlled upon binding of a small molecule called cyclic-di-GMP (c-di-GMP), a ubiquitous second messenger produced and degraded by diguanylate cyclase and phosphodiesterases, respectively. In *Gluconacetobacter xylinus*, it has been suggested that c-di-GMP binds to BcsB, promoting an allosteric change in the protein conformation that leads to its activation (Mayer et al. 1991). Recently, a PilZ domain believed to be part of the c-di-GMP binding protein has been identified in several bacterial cellulose synthases, including BscA, although direct evidence for c-di-GMP binding is still missing (Amikam and Galperin 2006). c-di-GMP is now known to antagonistically control the motility and virulence of single, planktonic cells, on the one hand, and cell adhesion and persistence of multicellular communities on the other (Jenal and Malone 2006; Romling and Amikam 2006).

<p>Genetic analyses performed mostly in *S. Typhimurium* revealed that the combined and coregulated syntheses of cellulose and curli fimbriae lead to a distinctive phenotype on Congo red agar plates, the red dry and rough (rdar) morphotype (Zogaj et al. 2001; Solano et al. 2002; Romling et al. 2003; Da Re and Ghigo 2006). While most of what is known of the regulation of cellulose production has been learned from *Salmonella*,

cellulose has also been found in *E. coli*, where cellulose synthesis is correlated with biofilm formation and expression of multicellular behavior (rdar morphotype), and where treatment with cellulase totally disperses existing biofilms (Zogaj et al. 2001, 2003; Romling 2002; Da Re and Ghigo 2006).

<heading4>Colanic Acid

<p>Colanic acid is a negatively charged polymer of glucose, galactose, fucose, and glucuronic acid that forms a protective capsule around the bacterial cell under specific growth and environmental conditions (for example, colanic acid is not produced in rich medium at 37°C). Colanic acid has a structure and assembly pathway very similar to that of the group I capsule and is therefore often included in that category. However, in contrast to most capsular types, a significant portion of the colanic acid produced is released into the extracellular medium.

<p>Colanic acid synthesis involves 19 genes located in the same cluster, named *wca* (formerly known as *cps*) (Stevenson et al. 1996). It is induced by the three-component system RcsC/RcsD/RcsB and requires an auxiliary positive transcription regulator RcsA (Majdalani and Gottesman 2005). Although the signal for sensor kinase RcsC remains uncharacterized, RcsC seems to respond to complex cues such as desiccation, osmotic stress, the level of periplasmic glucans, and growth on a solid surface (Ophir and Gutnick 1994; Sledjeski and Gottesman 1996; Ferrieres and Clarke 2003). A recent observation also indicates that colanic acid is induced by near-lethal levels of a subset of β-lactam antibiotics that may exacerbate the formation and persistence of a biofilm (Sailer et al. 2003). Although colanic acid has been reported to impair initial bacterial attachment, its synthesis is consistently upregulated within biofilms, and its production plays a role in the development of the mature biofilm architecture (Prigent-Combaret and Lejeune 1999; Prigent-Combaret et al. 1999; Danese et al. 2000b; Hanna et al. 2003). Interestingly, several groups have reported that expression of the colanic acid capsule may also have an inhibitory effect upon the biofilm ability of *E. coli* strains by masking autotransporter adhesins such as Ag43 and AidA (Hanna et al. 2003; Schembri et al. 2004).

<heading3>Cell Surface Polysaccharides

<p>Cell-surface glycoconjugates play a critical role in interactions between bacteria and their immediate environment. Besides released polysaccharides that have been identified as part of the biofilm matrix, surface polysaccharides can also contribute to the biofilm phenotype. Most *E. coli* isolates produce a complex layer of serotype-specific surface

polysaccharides: the lipopolysaccharide (LPS) O antigen and capsular polysaccharide K antigen. Variations in the structure of these polysaccharides give rise to 170 different O antigens and 80 K antigens that enable typing of most enterobacteria.

<heading4>Lipopolysaccharides

<p>The lipopolysaccharide (LPSs), also known as endotoxin, is a glycolipidic polymer that constitutes the main component of the outer leaflet of the outer membrane of Gram-negative bacterium. Constitutively expressed, LPS consists of three parts: lipid A, which is the toxic component and to which the core region is attached, which can be divided into an inner and an outer part; and finally the O-antigen polysaccharide, which is specific to each of the 170 *E. coli* serogroups.

<p>The sugar residues in lipid A and the core region are decorated to varying extents with phosphate groups and phosphodiester-linked derivatives, ensuring microheterogeneity in each strain. The lipid A part is highly conserved in *E. coli*, while the core contains six different basic structures, denoted R1–R6. The O-polysaccharide is linked to a sugar in the outer core. The O-antigen, absent in rough strains such as *E. coli* K-12, usually consists of 10–25 repeating units containing two to seven sugar residues. Thus, the molecular mass of the LPS present in smooth strains will be up to 25 kDa. Finally, in some serotypes, the core can be bound to group 1 capsule, forming K_{LPS} (Raetz 1996).

<p>More than 50 genes are required to synthesize LPS and assemble it at the cell surface. Some of these genes are clustered in large operons or are isolated on the *E. coli* chromosome. Mutations affecting LPS synthesis have been shown to affect *E. coli* ability to adhere to abiotic surfaces (Genevaux et al. 1999), suggesting a role of LPS in adhesion processes. Studies investigating *E. coli* mature biofilm formation also reported that LPS mutation could lead to a significant decrease in biofilm capacity. Inactivation of *waaG*, coding for a LPS core glycosyltransferase that resulted in a truncated LPS core structure and a deep rough phenotype, completely abolished biofilm formation of uropathogenic strain 536 without affecting its growth rate in liquid culture, suggesting that an intact LPS core is a major factor in adhesion to abiotic surfaces in *E. coli* strain 536 (Landini and Zehnder 2002; Beloin et al. 2006). However, since alteration of LPS synthesis can also impair Type 1 pili and colanic acid expression as well as bacterial motility, the phenotype of LPS mutants could still be attributed to indirect effects.

<p>In contrast, like capsule masks the function of short membrane adhesins, it has recently been reported that the reduction in LPS expression caused by an *rfaH* mutation

could unmask *E. coli* adhesins and therefore allow initial adherence and/or biofilm formation (Beloin et al. 2006).

<p>These results suggest two distinct mechanisms by which LPS either promotes or inhibits biofilm formation, mainly by interacting with cell-surface-exposed adhesion factors.

<heading4>Capsules

<p>*E. coli* capsules are surface-enveloping structures comprising high-molecular-weight capsular polysaccharides that are firmly attached to the cell (see, however, below). They are well-established virulence factors, often acting by protecting the cell from opsonophagocytosis and complement-mediated killing (Whitfield 2006). The 80 different capsular serotypes in *E. coli* were originally divided into more than 80 groups based on serological properties. Despite the diversity of bacterial capsule glycoconjugates and the complexity of their synthesis and assembly processes, later revisions classified capsules into four groups. *E. coli* group 1 and 4 capsules share a common assembly system, and this is fundamentally different from that used for group 2 and 3 capsules (Whitfield and Roberts 1999; Whitfield 2006).

<p>As seen for colanic acid and LPS, the *E. coli* capsule has also been shown to play an indirect role in biofilms by shielding of bacterial surface adhesin (Schembri et al. 2004). While capsular polysaccharides are linked to the cell surface of the bacterium via covalent attachments, capsule can be released into the growth medium as a consequence of the instability of phosphodiester linkage between the polysaccharide and the phospholipid membrane anchor (Roberts 1996; Whitfield 2006). Recently, group II capsular polysaccharides were shown to be significantly released into the culture supernatant and to display antiadhesion activities toward both Gram-positive and Gram-negative bacteria, therefore antagonizing biofilm formation by a mechanism distinct from steric hindrance of surface adhesin (Valle et al. 2006). Capsule-mediated biofilm inhibition is widespread in extraintestinal *E. coli*, suggesting that the anti-biofilm property of group II capsular polysaccharides could also play a role in the biology of these pathogens. Group II capsule may contribute to competitive interactions (bacterial interference) within bacterial communities, or to modulating *E. coli*'s own adhesion to surfaces encountered during the intestinal or urinary tract colonization process. Analyses showed that group II capsular polysaccharides affect biofilm formation by weakening cell-surface contacts (initial adhesion), but also by reducing cell–cell interactions (biofilm maturation).

Interestingly, direct treatment of abiotic surfaces with group II capsular polysaccharides drastically reduces both initial adhesion and biofilm development by important nosocomial pathogens, which may be used in the design of new anti-biofilm strategies (Valle et al. 2006).

<p>We have seen that *E. coli* biofilm initiation and maturation can involve many different factors. None of them, however, are strictly required. Indeed, *E. coli*'s ability to form a biofilm depends considerably on environmental conditions, and even well-demonstrated adhesion factors can be replaced by others. For instance, expression of conjugative pili totally overcomes the need for curli, type 1 pili of flagellar expression (Reisner et al. 2003). These results not only suggest that many different pathways can be used during *E. coli* biofilm formation, but also that regulatory mechanisms could coordinate the biofilm adhesion and maturation processes.

<heading1>Regulatory Events During Biofilm Development

<heading2>Predisposition to Surface Adhesion

<heading3>Phase Variation and Coordinated Expression of Surface Components

<p>In bacteria, the expression of many surface components is governed by a specific regulation mode called phase variation. This process induces the differential expression of one or several genes and leads to the emergence of two subpopulations within a clonal population either expressing (ON situation) or not expressing (OFF situation) factor(s) suggested to cause phase variation. In addition to its known role in protecting bacteria from the immune system, this mechanism of phase variation is now recognized to modulate exposure of cell surface components and especially adhesin molecules (van der Woude 2006). In *E. coli*, two major adhesins implicated in biofilm formation are subjected to this phase variation mechanism: type 1 fimbriae and the autotransported Ag43 adhesin.

<p>The promoter for the fimbrial subunit gene, *fimA*, lies within a short segment of invertible DNA known as the *fim* switch (*fimS*), and the orientation of the switch in the chromosome determines whether *fimA* is transcribed or not (Abraham et al. 1985). Inversion is catalyzed by two site-specific recombinases, the FimB and FimE proteins. The FimB protein inverts the switch in either direction, while FimE inverts it predominantly to the OFF orientation. When FimB and FimE are coexpressed, FimE activity dominates and the switch turns to the OFF phase (Klemm 1986; Dorman and Higgins 1987; Eisenstein et al. 1987; Gally et al. 1996; Blomfield et al. 1997). This dominance can be environmentally modulated, notably via DNA supercoiling modulation that modifies the

formation and stabilization of a recombination-proficient protein nucleocomplex encompassing several nucleoid-associated proteins such as IHF, H-NS, and Lrp (reviewed in van der Woude and Baumler 2004). Other regulators such as the LrhA protein, a regulator of the LysR family previously described as a repressor of flagellar motility (Lehnen et al. 2002), also influence phase variation of type 1 fimbriae. LrhA appeared to be a repressor of type 1 fimbriae both in K-12 and UPEC strains, mainly via its activation of the *fimE* recombinase gene (Blumer et al. 2005). Consequently, LrhA acts as a repressor of the initial phase of biofilm formation in *E. coli* (Blumer et al. 2005). Recently, *fimB*- and *fimE*-independent *fimS* phase variation of type 1 fimbriae has been identified both in meningitis-causing *E. coli* K1, by tyrosine site-specific recombinase HbiF (Xie et al. 2006), and in uropathogenic *E. coli* CFT073 by two recombinases encoded by *ipuA* and *ibpA* (Bryan et al. 2006).

<p>The expression of Ag43 is also phase-variable and represents a classical example of bacterial bi-stability. The shift from $Ag43^{+}$ to $Ag43^{-}$ cells is governed by a mechanism involving the concerted action of both the Dam GATC DNA-methylating enzyme deoxyadenosine methylase (activation) and the transcriptional regulator OxyR (repression) (Owen et al. 1996; Henderson et al. 1997b; Wallecha et al. 2002). Three GATC sites are present in the promoter region of the *flu* gene, two of which overlap with an OxyR binding site centered at position +37 from the +1 of transcription. In a wild type situation, each bacterium is either in an Ag43 OFF situation, if, after DNA replication, the OxyR protein manages to bind to its consensus site before DNA methylation, thus stopping RNA polymerase progression, or in an Ag43 ON situation, if DNA methylation occurs before OxyR can bind to DNA. This ON or OFF state of each bacterium is reflected by, respectively, the presence or absence of Ag43 at the cell surface.

<p>The studies presented above clearly indicate that complex regulation mechanisms are used by bacteria to modulate expression of different factors required for biofilm formation, especially when these factors are required at different times in biofilm development. For example, type 1 fimbriae are important in the initial steps of bacteria-to-surface interactions, whereas Ag43 is required later to promote bacteria-to-bacteria interactions. In addition to the individual mechanisms of phase variation, several mechanisms of coordinated regulation between different surface components involved in adhesion have been reported (Holden and Gally 2004). Among coordinated regulation implicating different adhesins, flagella, and capsules, the best characterized is the coordination between type 1 fimbriae and Ag43 production. Schembri and co-workers first showed that overexpression of type 1 fimbriae (and also of P or F1C fimbriae) abrogates

exposure of Ag43 to cell surfaces (Schembri and Klemm 2001). This effect is mediated by a regulatory effect at the Ag43-encoding gene, *flu,* whose quantity of RNA transcript is enhanced 20-fold when genes encoding type 1 fimbriae are absent (Schembri et al. 2002). Fimbriation does not affect Ag43 production in an *oxyR* background in which the *flu* promoter is in an ON situation (Schembri and Klemm 2001). Until recently, it was presumed that *flu* was only repressed by the reduced form of OxyR (Henderson et al. 1999; Haagmans and van der Woude 2000). Two results suggest that a modification in the redox status of OxyR could explain the effect of type 1 fimbriae expression on Ag43 production: first, the addition of the reducing agent DTT counteracts the effect of deletion of type 1 fimbriae; second, overproduction of flagella, which do not contain any disulfide bonds, has no effect on Ag43 exposure at the cell surface (Schembri and Klemm 2001). This suggests that expression of organelles containing disulfide bonds, such as type 1, P or F1C fimbriae, could affect cellular thiodisulfide status and thus modify the redox status of OxyR and the expression of Ag43. However, this hypothesis is not consistent with recent indications that phase variation in Ag43 is independent of the oxidation status of OxyR (Wallecha et al. 2003). However, in addition to its role in biofilm formation, Ag43-mediated autoaggregation seems to protect cells from oxidizing agents (Schembri et al. 2003a). Moreover, independently of Ag43 expression, the presence of fimbriae on the cell surface seems to abrogate the intercellular Ag43–Ag43 interaction that is required for autoaggregation to occur (Hasman et al. 1999). The reciprocal is not true, and Ag43 or OxyR status does not appear to influence fimbriae expression (Hasman et al. 1999). Hence, the correlation between Ag43 expression, type 1 pili, and OxyR status remains to be clarified.

<p>The presence of type 1 pili is not the only extracellular component to interfere with Ag43 activity. A recent study demonstrated that the presence of capsules such as K1 or K5 capsules could block Ag43 functionality (Schembri et al. 2004). Whereas type 1 pili production interferes with both Ag43 expression and interaction of Ag43 molecules, capsule production appears solely to sterically shield the Ag43–Ag43 interaction. As a consequence, encapsulated cells expressing both capsule and Ag43 are impaired in biofilm formation on a polystyrene abiotic surface compared with nonencapsulated cells.

<p>A recent study by Ulett and co-workers demonstrated that Ag43-mediated autoaggregation impaired motility (Ulett et al. 2006), thus reinforcing the idea that *E. coli* has developed specific mechanisms to coordinately express different surface components required at different stages of biofilm formation.

<heading3>Fertility Inhibition System

<p>Most of the genes required for conjugative transfer of F plasmid DNA are encoded by the 33-kb transfer (tra) operon of F-like conjugative plasmids (Frost et al. 1994). Transcription of the *tra* operon is positively regulated by the TraJ transcriptional activator which, in turn, is negatively regulated by the FinOP fertility inhibition system. The FinOP system consists of an antisense RNA, FinP, and a 21.2-kDa protein, FinO, which together inhibit TraJ expression. Except for the F-plasmid itself, whose *finO* gene is interrupted by an IS3 insertion element, in F-like plasmids the regulatory activity of FinP depends upon the action of the plasmid-encoded protein, FinO (Yoshioka et al. 1987; van Biesen and Frost 1992). However, this fertility inhibition mechanism is not completely controlled, thus leaving in a planktonic population of F-like-bearing bacteria a low proportion of bacteria expressing conjugation function. These piliated bacteria are therefore prompted to easily integrate into a preformed biofilm and to transmit their conjugative plasmid to the recipient population, thus promoting maturation of the biofilm. Hence, this fertility inhibition mechanism may help bacteria to switch from a planktonic to a sessile mode of growth.

<heading2>Regulatory Events upon Initial Interaction with Surfaces

<p>Environmental conditions existing on immerged surfaces are different from those found in the surrounding medium. For example, the formation of a conditioning film on the surface modifies nutriment concentrations as well as other physicochemical factors such as pH, oxygenation, and osmolarity. How bacteria know that they are on a surface is still poorly understood. Among possible inducing cues are direct physical contact, perception of extracellular signals, or gradients and bacteria-to-bacteria interactions (Harshey and Toguchi 1996) that allow the bacteria to produce productive cell–surface interactions and therefore lead to the formation of stable biofilm. *E. coli* has proven to be a valuable model for investigating some aspects of these questions.

<heading3>Sensing the Surface: Regulation of Genes upon Direct Contact of Bacteria with Surfaces

<heading4>The *cpx* System Senses the Surface and Neighboring Bacteria

<p>The two-component regulatory *cpxRA* system is composed of the sensor membrane protein CpxA and of the cytoplasmic regulator CpxR. This system is known to respond to envelope stresses such as overproduction and misfolding of membrane proteins and elevated pH (Raivio and Silhavy 2001) and notably to activate the expression of genes encoding protein chaperones or proteases such as *dsbA* and *degP*. Activation of the *cpx*

pathway therefore participates in the adaptation of bacteria to environmental stresses. Early adhesion of *E. coli* cells to abiotic surfaces, probably by inducing membrane perturbation, has been shown to activate the *cpx* system through a process called surface sensing (Otto and Silhavy 2002). This abiotic surface contact induction depends on CpxR, the cognate sensor of the system, but also on NlpE, an outer membrane lipoprotein previously shown to induce the *cpx* system when overproduced (Snyder et al. 1995). A mutation in the *cpxR* gene alters cell–surface interactions (Otto and Silhavy 2002). This suggests that the *cpx* system is required for an adaptive response necessary for stabilizing contact of attached cells with the surface. Among responses of the *cpx* system to surface contact are modifications in cell-surface composition through regulation of cell-surface protein expression (i.e., OmpC) (De Wulf et al. 2002) and modulation of flagellar gene expression, since *cpxR* is a repressor of motility and chemotaxis genes (De Wulf et al. 2002), two pathways that must be switched off to optimize initial attachment and to avoid leaving the recently colonized surface. Flagellar encoding genes are consistently repressed early after bacteria reach the surface (Prigent-Combaret and Lejeune 1999; Ren et al. 2004a), and overexpression of flagellar genes, either directly or indirectly via disruption of flagellar gene repressors such as *hha* / *ybaJ* genes, reduces biofilm formation by *E. coli* (Tenorio et al. 2003; Barrios et al. 2006). In addition to sensing the surfaces, the *cpx* system may also sense neighboring bacteria. Components of the *cpx* system are indeed induced in mature *E. coli* biofilms, where most of the bacteria are in contact with one another rather than with the surface (Beloin et al. 2004). Induction of the *cpx* pathway may therefore play an important role in maturation of biofilm by affecting both initial adhesion between bacteria and the surface and subsequent interactions between the bacteria themselves (Otto and Silhavy 2002; Beloin et al. 2004). Since the *cpx* pathway, when activated, also impairs biogenesis of proteinaceous adhering appendages such as curli or F-pili, Dorel and co-workers recently proposed that the role of this system in each newly divided cell in a biofilm might well be to turn down expression of such energy-costly adhering molecules right after productive interactions are secured between the bacteria and surfaces, and between bacteria themselves (Dorel et al. 2006).

<heading4>The *rcs* System Mediates Biofilm Maturation

<p>The *rcs* two-component regulatory pathway is composed of membrane-associated proteins RcsC and RcsD and the cytoplasmic response regulator RcsB. Rcs phosphorelay in *E. coli* was originally described as being a regulator of *cps* operon

expression, encoding proteins required for production of capsular polysaccharide colanic acid (for review see Majdalani and Gottesman 2005; Huang et al. 2006). Although the exact signals sensed by RcsC remain incompletely characterized, several studies have shown that this sensor kinase responds to complex signals, including desiccation and changes in osmolarity (Ophir and Gutnick 1994; Sledjeski and Gottesman 1996). More recently, the RcsC sensor kinase has been shown to respond to growth on a solid surface by sensing membrane perturbations and therefore to be required for normal biofilm development in *E. coli* (Ferrieres and Clarke 2003). Ferrières and co-workers demonstrated that surface induction of colanic acid genes observed in *E. coli* (Prigent-Combaret et al. 1999, 2000) depended on the *rcs* pathway, which also represses expression of surface appendages such as curli, fimbrial proteins, and Ag43 (Ferrieres and Clarke 2003). Considering that flagellar gene expression in *E. coli* is repressed by the *rcs* two-component signaling pathway as well (Francez-Charlot et al. 2003), induction of the *rcs* system is likely to cause remodeling of the bacterial surface. Moreover, the fact that initial attachment of cells is not decreased in an *rscC* or *rcsB* mutant (Danese et al. 2000b) suggests that this regulon plays a role in a later step, i.e., biofilm maturation, where colanic acid production, in particular, is necessary for proper architecture of the biofilm, whereas flagella need to be repressed.

<p>Recent studies on modifications in the composition of outer membrane proteins during fimbriae-mediated adhesion also demonstrated that such cell–surface interactions indeed induce remodeling of the bacterial envelope (Otto et al. 2001). Among proteins whose the quantity is reduced upon attachment to abiotic surfaces is the OmpX protein (see Sect. 2.3.1) (Otto and Hermansson 2004).

<p>Consequently, it appears that contact with a solid surface induces an adaptive response in *E. coli* cells leading to stable adhesion. This surface-sensing mechanism appears to involve several pathways, whose complex overlapping and interplay need to be elucidated.

<heading3>Sensing Microenvironments at an Abiotic Surface: The EnvZ/OmpR Two-Component Pathway Senses Surface Osmolarity

<p>As stated earlier in this chapter, surfaces tend to adsorb organic molecules and to acquire a conditioning film that locally modifies osmolarity compared with the surrounding medium. Bacteria entering into contact with surfaces will therefore face a favorable growth environment, especially if the surrounding medium contains low nutrient concentrations. It has been proposed that this growth advantage may be a significant

selective force driving growth on abiotic surfaces (Costerton et al. 1987). The EnvZ/OmpR two-component pathway is known to respond to external osmolarity by regulating transcription of the *ompF* and *ompC* porin-encoding genes; moreover, it increases surface adhesion in response to moderate increases in osmolarity (Prigent-Combaret et al. 2001). This EnvZ/OmpR activity may thus favor adhesion in zones supporting high metabolic activity, both by repressing flagellar gene expression (Shin and Park 1995; Oshima et al. 2002) and by activating curli expression upon initial adhesion. The OmpR effect on curli expression is mediated by activation of the regulator CsgD by phosphorylated OmpR. Interestingly, *csgD* encodes a key regulator of the FixJ family that positively regulates production of curli and cellulose in *E. coli* and *Salmonella*, and these two factors promote biofilm formation (see Zogaj et al. 2001; Solano et al. 2002). Recently, modulation of *csgD* expression by modification of osmolarity was shown to be essentially dependent on the interplay between the two-component systems EnvZ/OmpR and CpxA/CpxR and the histone-like protein H-NS (Jubelin et al. 2005).

<p>A recent study identified the gene *bolA* to be a potential regulator of biofilm formation. *bolA* has been described as being negatively regulated by OmpR (Yamamoto et al. 2000). It is involved in *E. coli* morphogenesis and was shown, when overexpressed, to cause round-cell morphology (Aldea et al. 1988) and to increase the ratio of OmpC/OmpF porins, leading to a subsequent decrease in cell membrane porin-mediated permeability (Freire et al. 2006). Furthermore, the morphological effect of *bolA* overexpression depends on an active *ftsZ* gene product (Aldea et al. 1988). *bolA* has been described as a regulator of cell-wall biosynthetic and biosynthesis enzymes (Santos et al. 2002). In minimal medium, mutation of *bolA* slightly reduces *E. coli* biofilm formation in 96-well polystyrene microtiter plates, whereas overexpression of *bolA* strongly induces biofilm formation (Vieira et al. 2004). These results hint at a physiological connection between cell morphology, cell division, and biofilm formation.

<heading3>A Role for Small Molecules in the Switch from Planktonic to Sessile Life

<heading4>Cyclic di-GMP

<p>Cyclic di-GMP (c-di-GMP) is a second messenger whose synthesis is performed by diguanylate cyclases, proteins bearing GGDEF motifs, whereas its degradation is ensured by proteins containing EAL domains and with phosphodiesterase activity (Romling et al. 2005). *E. coli,* like most bacteria, contains multiple diguanylate cyclases and phosphodiesterases with as many as 19 GGDEF proteins and 13 EAL proteins detected in its genome (Romling et al. 2005). It is likely, therefore, that localization of

these proteins in the bacterial cell is a key factor enabling fine-tuning of c-di-GMP concentrations in different areas of the cell. c-di-GMP appears to act posttranscriptionally by activation of specific proteins through direct interactions, as has been shown for the protein complex containing the cellulose synthase BcsA of *Gluconacetobacter xylinus* (Amikam and Benziman 1989). A domain called PilZ was recently bioinformatically identified in several GGDEF and/or EAL proteins, and notably in the BcsA and YcgR proteins in *E. coli*, as being a possible domain of interaction with c-di-GMP (Amikam and Galperin 2006). Among numerous cellular functions linked to c-di-GMP is biofilm formation. In *E. coli*, as in other bacteria, a correlation has been demonstrated between a high c-di-GMP concentration and biofilm formation vs a low c-di-GMP concentration and motility (Simm et al. 2004). Cellulose synthesis and rotation of flagella are the main functions responsible for such correlations. Ko and Park showed that expression of the EAL domain protein YhjH is required to stimulate swimming motility in a *hns* mutant of *E. coli,* whereas swimming motility was stimulated by deletion of *ycgR* that encodes a PilZ domain protein (Ko and Park 2000). One hypothesis is that a YcgR–c-di-GMP complex inhibits the motor function of the flagella, whereas degradation of cylic-di-GMP by YhjH relieves inhibition of motility by YcgR (Romling and Amikam 2006). Extrapolating from the work done in *Salmonella* on regulation of cellulose production (Zogaj et al. 2001, 2003; Romling 2002; Solano et al. 2002), activation by the regulator CsgD of expression of *adrA*, a diguanylate cyclase-encoding gene, was thought to be the unique pathway employed by *E. coli* to activate cellulose production. However, recent studies demonstrate that in *E. coli* the mechanism of regulation of cellulose synthesis is more complex than previously thought. Brombacher and co-workers showed that in *E. coli* K-12 MG1655, in addition to *adrA*, CsgD also activated *yoaD*, a putative phosphodiesterase-encoding gene whose mutation consistently activates cellulose production. Consequently, in this strain, cellulose production might be regulated by subtle modifications in c-di-GMP concentrations resulting from AdrA- and YoaD *csgD*-dependent activation (Brombacher et al. 2006). A recent study also demonstrated that, in addition to being regulated by RpoS through its action on MlrA (whose mechanism of function is unknown) (Gerstel et al. 2003), two RpoS-regulated GGDEF and/or EAL proteins, YdaM and YciR, inversely regulate *csgD* expression, thus influencing both curli and cellulose production (Weber et al. 2006). The expression of genes encoding these two proteins was also inversely regulated by H-NS, again increasing the complexity of the mechanisms governing *csgD* regulation (Weber et al. 2006). Moreover, Da Re and Ghigo showed that, alongside a *csgD*- and *adrA*-dependent mechanism of cellulose synthesis identified in the

enteroaggregative strain 55989, a *csgD*-independent pathway leads to cellulose synthesis in the commensal 1094 and probiotic Nissle 1917 (DSM6601) strains, whereas *csgD* activation of curli production is maintained (Da Re and Ghigo 2006). This pathway, instead of using *adrA*, passes through expression of another putative diguanylate cyclase called YedQ (Da Re and Ghigo 2006). Furthermore, in another *E. coli* commensal isolate 1125, cellulose production appeared to be independent of *csgD*, *adrA* and *yedQ*, thus suggesting that an as yet uncharacterized GGDEF protein distinct from both AdrA and YedQ may have acquired this cellulose regulatory function.

<heading4>Acetyl Phosphate

<p>Among small molecules that coordinate gene expression in response to environmental stimuli, two molecules have recently been identified as signals linking nutrient status to biofilm formation in *E. coli*: acetyl phosphate (AcP), which accumulates intracellularly in the presence of an abundant carbon source and/or a low oxygen concentration in the medium (Wolfe et al. 2003), and ppGpp, the molecule of the stringent response that accumulates upon nutrient starvation conditions (Balzer and McLean 2002) (see below). Local depletion of oxygen occurring when bacteria reach a surface is hypothesized to be the signal causing intracellular AcP levels to rise. An increase in AcP levels correlates notably with an elevated level of type 1 pili and colanic acid gene expression, and with a decreased level of flagellar gene expression, favoring maturation of the biofilm (Pruss and Wolfe 1994; Wolfe et al. 2003). One hypothesis is that AcP could influence biofilm formation by acting as a phosphodonor for response regulators such as FimZ, OmpR, and RcsB, known to control biofilm-associated genes. Fredericks and co-workers determined that AcP indeed exerts its effect on capsular and flagellar gene expression completely or partially by means of the Rcs phosphorelay (Fredericks et al. 2006). In addition, as in the surface-sensing mechanisms described above, modifications in the intracellular AcP pool could also lead to bacterial surface modifications. Indeed, genes that respond negatively to high AcP levels include these encoding outer membrane porins (*ompF*, *ompC*) and other proteins associated with, or predicted to be associated with, the envelope (*rbsB*, *b1996*, *yqiH*, *glpD*, *rfbX*, *rbsD*) (Wolfe et al. 2003).

<heading4>The Alarmone ppGpp

<p>The effect of ppGpp on biofilm formation was assessed by comparing wild type *E. coli* K-12MG1655 with an isogenic *relA spoT* mutant that does not produce ppGpp (Balzer

and McLean 2002). Clearly, in nutrient-limited conditions, the absence of ppGpp production caused a decrease in biofilm cell density, therefore signifying that the stringent response is necessary for normal development of *E. coli* biofilm. However, in rich LB medium where the ability to synthesize ppGpp is also crucial, the reverse was observed, i.e., the absence of ppGpp production caused an increase in biofilm cell density (Balzer and McLean 2002). A recent work by Aberg and co-workers seems to contradict this result, since they showed that in LB broth, the absence of ppGpp reduced biofilm formation in uropathogenic and K-12 *E. coli* strains (Aberg et al. 2006). Furthermore, Aberg and co-workers showed that ppGpp promoted biofilm formation via increased type 1 fimbriae expression due to activation of expression of the *fimB* recombinase gene. This ppGpp effect is, in fact, independent of RpoS, H-NS, and NanR, three previously described regulators of *fimB* expression (Aberg et al. 2006). The authors proposed that induction of intracellular ppGpp levels can be considered as an alert signal that promotes expression of genes, including those for type 1 fimbriae, which could both adapt cells to slow growth and increase the probability of surviving stressful environments, two situations encountered by bacteria within biofilm. It can also be envisaged that ppGpp signaling in planktonic bacteria growing in nutrient-depleted media, and via an increase in type 1 fimbriae production, may favor colonization of surfaces in which conditions are more advantageous.

<heading4>N-Acetyl-Glucosamine and N-Acetylglucosamine-6-P

<p>*E. coli* can use N-acetylglucosamine-6-P (GlcNAc-6P) as a carbon source and/or as a precursor for peptidoglycan and lipopolysaccharide biosynthesis. GlcNAc-6-P is obtained either by phosphorylation of N-acetyl-glucosamine (GlcNAc) acquired from the environment or by de novo synthesis. When high levels of GlcNAc-6P accumulate in the cell, the repression exerted by the protein NagC on *nag* genes (*nagABCDE*) is relieved and these genes then support utilization of GlcNAc as a carbon source. Barhnart and co-workers found that curli gene expression is repressed in response to artificially elevated levels of intracellular GlcNAc-6-P created by deletion of *nagC* or *nagA*, a deacetylase that converts GlcNAc-6-P to glucosamine-6-phosphate (GlcN-6P) (Barnhart et al. 2006). Production of another adhesive fiber, type 1 fimbriae, was previously shown to be activated by GlcNAc-6-P (Sohanpal et al. 2004). This later effect appeared to be NagC-dependent through activation of *fimB* recombinase gene expression that switches the expression of type 1 fimbriae from OFF to ON. In addition to its role as a carbon source, GlcNAc-6-P might well be a signal for the cell to produce adhesive molecules.

Interestingly, *E. coli* produces an extracellular polysaccharide composed of the GlcNAc subunit (the PGA) that promotes attachment to solid surfaces, cell–cell adherence, and stabilization of biofilm structure (Wang et al. 2004; Itoh et al. 2005). However, a possible link between PGA production and regulation of cell surface molecules promoting adhesion remains to be elucidated.

<heading2>Genetic Regulation Within *E. coli* Biofilms

<heading3>Gene Expression Within Biofilm: Cause or Consequence?

<p>Planktonic and surface-attached growth modes are simple to distinguish phenotypically. These two lifestyles are thought to require or involve a different gene expression setup, leading to the expression of some of the phenotypic characteristics of the biofilm phenotype. The existence of changes in gene expression within biofilm compared with a non-biofilm mode of growth was recognized early on. Gene fusion studies suggest that the expression of up to 38% of the *E. coli* genome is affected by biofilm formation (Prigent-Combaret et al. 1999). Such evidence for differential gene expression within bacterial biofilms has been provided by recent studies using DNA arrays. These studies indicate that, in fact, a lower proportion of the *E. coli* genome (5%–12%) is subject to differential expression in sessile vs planktonic life (Schembri et al. 2003b; Beloin et al. 2004; Ren et al. 2004a). While these studies (along with others conducted in other microorganisms) suggest the existence of a common pattern of gene expression in *E. coli* biofilms and have indeed identified some genes required for biofilm formation, detailed comparison of genes discovered reveal only a very modest overlap between the different studies. This underscores the difficulty in comparing analyses carried out with different strains, different experimental setups (biofilm device, medium, presence or absence of flow) and different time scales (i.e., with different *E. coli* biofilms), but it also raises the possibility that global analyses are not really appropriate for dealing with the extreme complexity in time and space that resides within a biofilm (Beloin and Ghigo 2005).

<p>Moreover, it is important to distinguish between factors required for biofilm formation and factors induced by particular biofilm conditions. Indeed, although biofilm formation requires the expression of specific factors (see above), major modifications in gene expression patterns within biofilms could also be induced by the drastic environmental changes occurring during biofilm formation. Biochemical and genetic evidence support the hypothesis that bacteria face different conditions within a biofilm as compared with planktonic growth (Huang et al. 1998; Prigent-Combaret et al. 1999, 2001). Indeed,

biofilm bacteria are likely to be subjected to progressive microaerobic conditions, increased osmotic pressure, pH variation and decreased nutrient accessibility. These biofilm conditions often have strong similarities with conditions that prevail in stationary phase (planktonic) cultures, and when the stationary phase character of the bacterial lifestyle within biofilm has been investigated, it has generally been shown that a significant part of the *E. coli* K-12 biofilm response involves stationary-phase-induced genes (Schembri et al. 2003b; Beloin et al. 2004). Since many changes observed in biofilm gene expression are potentially a consequence rather than a cause of biofilm formation, the question as to whether these genes encode functions that are required by, or that are induced by, biofilm conditions remains to be determined.

<heading3>Regulation of Biofilm Formation by Central Carbon Flux

<p>Catabolite repression has recently been recognized to be a regulatory signal controlling *E. coli* biofilm formation (Jackson et al. 2002a). The presence of 0.2% glucose in rich medium appears to decrease biofilm biomass. However, this effect is more pronounced when glucose is added during the initial steps of biofilm formation rather than in later stages of biofilm maturation, suggesting that catabolite repression preferentially affects components required in the early stages of bacterial adhesion (Jackson et al. 2002a). Glucose repression is partially mediated by the cAMP receptor protein CRP. Indeed, a *crp* mutant displays decreased biofilm formation abilities compared with the wild type (Jackson et al. 2002a). Recently, Domka and co-workers identified two biofilm-induced genes, *yceP* and *yliH* (renamed *bssS* and *bssR* for regulator of biofilm through signal secretion) (Schembri et al. 2003b; Beloin et al. 2004; Ren et al. 2004a), which appear to be key regulators of several genes involved in catabolite repression and could participate in the negative effect of glucose on *E. coli* biofilm formation (Domka et al. 2006). Mutations in these two genes increased biofilm formation only in the presence of glucose and could possibly reduce both phosphorylation and transport of glucose (Domka et al. 2006). BssS (YceP) and BssR (YliH) could notably repress biofilm formation via different systems modulated by glucose, implicating regulators such as RpoS, CRP, CreC, and CsrA (Domka et al. 2006). These two genes were also recently identified as biofilm-induced when two asymptomatic uropathogenic *E. coli* strains (83972 and VR50) were grown in urine (Hancock and Klemm 2006). As opposed to results by Domka and co-workers (2006), the mutation of *yceP* in this study led to decreased biofilm formation, as previously observed in a study on biofilm formation of the F plasmid-bearing strain TG1 (Beloin et al. 2004). Therefore, these two genes, and especially *yceP,* appear to play

differential roles in biofilm formation depending on the growth conditions and strains used. <p>The CsrA protein has been extensively studied in recent years. This protein has been shown to repress biofilm formation (Jackson et al. 2002b). Until recently, CsrA was thought to affect biofilm formation only through repression of glycogen metabolism and its regulatory effect on the swimming-motility master regulator *flhDC* (Wei et al. 2001). Expression of *csrA* is indeed sharply decreased a few hours after initiation of growth on surfaces, a profile that is compatible with the decrease in flagellar gene expression upon attachment. On the other hand, *csrA* expression is reactivated after maturation of the biofilm (2-day-old biofilm). An increase in *csrA* expression in mature *E. coli* biofilm might also lead to resumption of swimming motility. Therefore, there may be a link between increased flagellar gene expression and biofilm detachment after reinitiation of swimming motility, a hypothesis found in early work on motility and biofilm (Pratt and Kolter 1998). In line with this, Jackson and co-workers showed that overexpression of *csrA* was responsible for biofilm dispersal (Jackson et al. 2002b). Lately, Wang and co-workers have shown that the biofilm effect exerted by CsrA is, in fact, results essentially from its effect on production of the polysaccharide adhesin PGA (*pgaABCD*), with a deletion of *csrA* having no effect on biofilm formation in a Δ*pgaC* mutant (Wang et al. 2004, 2005). CsrA directly affects PGA production at a posttranscriptional level and may indirectly affect *pgaABCD* expression via its effect upon an as yet unidentified regulator of the *pgaABCD* operon (Wang et al. 2005). Two untranslated RNAs, CsrB and CsrC, antagonize CsrA activity by sequestering this protein; consequently, deletion of either *csrB* or *csrC* represses biofilm formation (Wang et al. 2005). The Csr (carbon storage regulatory) system also involves the UvrY protein, the cognate regulator of the two-component system BarA/UvrY (Suzuki et al. 2002; Sahu et al. 2003). Whereas CsrA is necessary for UvrY activity, UvrY in turn activates *csrB* expression, thus implementing expression of the CsrA/CsrB/CsrC negative regulatory loop (Suzuki et al. 2002). Mutation of either *barA* or *uvrY* attenuates biofilm formation, suggesting that BarA and UvrY are necessary for development of a biofilm (Suzuki et al. 2002; Sahu et al. 2003). Besides the high number of genes regulated by UvrY (Oshima et al. 2002), the effect of UvrY on biofilm formation is directly linked to its role on CsrB/CsrC and disappears in a mutant that is unable to synthesize PGA (Wang et al. 2005). Recently, a new activator of *pgaABCD*, and thus biofilm formation, was discovered: the LysR-type-positive regulator NhaR that seems to activate this operon specifically in response to increased Na^+ concentration or pH (Goller et al. 2006).

<heading3>Quorum-Sensing Molecules Regulate *E. coli* Biofilm Formation

<heading4>SdiA, a Homoserine Lactone, Activates UvrY and Biofilm Formation

<p>While *E. coli* is not known to synthesize *N*-acylated homoserine lactones (AHL) (Ahmer 2004) and has no apparent AHL synthase in its genome, it contains the *sdiA* gene that encodes a protein of the LuxR family. LuxR proteins possess one domain for binding *N*-acylated homoserine lactones and a second domain for binding DNA. An *sdiA* mutant has been shown to produce threefold less biofilm than a wild type *E. coli* strain (Suzuki et al. 2002). This effect appears to be mediated by SdiA activation of the *uvrY* gene (Suzuki et al. 2002), and consequently, predominantly by the CsrB/CsrC untranslated RNA effect on CsrA. While the environmental signal that permits SdiA of *E. coli* to regulate *uvrY* expression remains to be determined, a study by van Houdt and co-workers showed that, at 30°C, *E. coli* responds in an SdiA-dependent manner to the addition of AHL by modification of expression of 15 genes, including upregulation of *uvrY* (Van Houdt et al. 2006). Also consistent with these results is the increased expression of *sdiA* in mutants of either *yceP* or *yliH*, whose deletion caused an increase in *E. coli* biofilm formation (Domka et al. 2006). This leaves us with the possibility that the biofilm formation abilities of *E. coli* can potentially be modulated by quorum-sensing AHL signaling molecules from other species, eventually interacting with *E. coli* in natural environments.

<heading4>The AI-2 Signaling Molecule Modulates *E. coli* Biofilm Formation

<p>*E. coli* strains do secrete the autoinducer-2 (AI-2) quorum-signaling molecule that is encoded by genes of the *luxS* family and that has been regarded as a universal cell–cell communication signal (Xavier and Bassler 2003). An *lsr*-like transporter system has been described recently in *E. coli* K-12 and this could serve to internalize AI-2 molecules (Xavier and Bassler 2005). Nor does disruption of the AI-2 signaling system of *E. coli* appear to modify biofilm maturation mediated by derepressed IncF plasmids in a flow-cell system (Reisner et al. 2003). A *luxS* mutation either did not affect or only moderately reduced the initial adhesion steps in biofilm formation on a microtiter plate (Colon-Gonzalez et al. 2004; Gonzalez Barrios et al. 2006). However, a furanone-based molecule that inhibits the *E. coli* AI-2 signaling system has been shown to decrease the thickness of *E. coli* biofilm formed on steel coupons or on air–liquid interfaces, and to increase the percentage of dead cells within the same biofilm (Ren et al. 2001, 2004b). Consistently, Gonzalez Barrios and co-workers showed that the addition of AI-2 activated *E. coli* biofilm formation through a complex regulatory cascade where MqsR (motility

quorum-sensing regulator), encoded by a biofilm-induced gene (Ren et al. 2004a), induced the flagellar operon activator two-component system QseBC (Sperandio et al. 2002) that in turn activated *E. coli* swimming motility (Gonzalez Barrios et al. 2006). In the presence of glucose, YdgG, another protein encoded by a biofilm-induced gene (Ren et al. 2004a) and renamed TqsA (transport quorum-sensing A), may participate in this regulatory cascade by exporting AI-2 molecules outside the cells, as well as the two genes *bssS* (*yceP*) and *bssR* (*yliH*) that are implicated in catabolite repression of the *lsr* operon that imports AI-2 into the cells (Domka et al. 2006). Indeed, a deletion of *ydg*G leads, in LB + glucose, to increased biofilm formation and, in different media, to an increase in intracellular levels of AI-2 (Herzberg et al. 2006). Furthermore, YdgG was found to repress cell surface determinants (genes related to flagellum, type 1 fimbriae, Ag43, curli, and polysaccharide production), as well as 10 genes newly recognized as important for *E. coli* biofilm formation (*yfjR*, *bioF*, *yccW*, *yjbE*, *yceO*, *ttdA*, *fumB*, *yjiP*, *gutQ*, and *yihR*),,and it appears to control these genes through AI-2 transport (Herzberg et al. 2006).

<heading4>Indole

<p>Indole production is a phenotypic trait displayed by several Gram-negative bacteria including *E. coli*. Indole is produced by the degradation of tryptophane, a reaction performed by tryptophanase encoded by the *tnaA* gene (Newton and Snell 1964). Indole has been described as a potential extracellular signal (Wang et al. 2001). Genes necessary for indole production (including *tnaA*) have been shown to be induced by addition of *E. coli* stationary-phase supernatant (Ren et al. 2004c), suggesting the existence of complex cross-talk between different extracellular signaling pathways. A mutant of *E. coli* K-12 S17-1 for gene *tnaA* is unable to produce a biofilm in 96-well polystyrene microtiter plates in LB medium (Di Martino et al. 2002). Addition of exogenous indole has no effect on biofilm formation of S17-1 itself (as shown also for *E. coli* K-12 MG1655 strains; Bianco et al. 2006), but restores normal biofilm formation in S17-1Δ*tnaA* (Di Martino et al. 2003). Moreover, whereas oxindolyl-L-alanine, a specific inhibitor of tryptophanase, has no effect on biofilm development of *K. pneumoniae*, an indole nonproducing species, it has a dose-dependent inhibitory effect on biofilm development of S17-1 and also of other indole-producing species such as urinary isolates of *E. coli*, *K. oxytoca*, *C. koseri*, *P. stuartii*, and *M. morganii* grown in LB or synthetic urine (Di Martino et al. 2003). A link between indole and AI-2 pathways has been recently pointed out by Herzberg and co-workers and Domka and co-workers (Domka et al. 2006;

Herzberg et al. 2006). In LB + glucose, a mutation of *tqsA* (*ydgG*) that exports AI-2 seems to activate the expression of the AcrEF multidrug efflux pump that exports indole outside the cells (Herzberg et al. 2006). Moreover, in LB + glucose and not in LB, mutation of *bssS* (*yceP*) or *bssR* (*yliH*), which also regulates AI-2 concentration, strongly reduces both intra- and extracellular concentrations of indole in *E. coli* K-12 BW25113, and at the same time is responsible for induction of expression of *acrE* and *acrF* and for repression of *mtr*, encoding pumps that both export and import indole (Domka et al. 2006). These authors conclude that *bssR* (*yliH*) and *bssS* (*yceP*) mutants increase biofilm formation by repressing indole concentrations through a catabolite repression-related process; they infer that indole represses *E. coli* biofilm formation, a conclusion that appears to be in conflict with results of Di Martino and co-workers (Di Martino et al. 2002, 2003). Given the differences in the media (LB or synthetic urine versus LB + glucose) and strains used (S17-1 and other Gram-negative bacteria vs BW25113), as well as the types of mutants analyzed (*tnaA* vs *bssR* and *bssS*), the role of indole in biofilm development appears to depend considerably on the conditions used (as does the role of *yceP* and *yliH*), and therefore remains to be elucidated.

<heading4> *O*-Acetyl-l-Serine, Another Extracellular Signal, Regulates *E. coli* Biofilm Formation

<p>Another diffusible molecule, *O*-acetyl-l-serine (OAS), appears to modulate *E. coli* biofilm formation. A mutation in the gene coding for a serine acetyltransferase *cysE*, which catalyzes the conversion of serine to *O*-acetyl-l-serine, was shown to enhance biofilm formation through reduction of the amount of an extracellular signal molecule. The authors suggest that OAS or other cysteine metabolites may play a physiological role, possibly by activating genes whose expression leads to inhibition of biofilm formation (Sturgill et al. 2004).

<heading3>Regulation of Biofilm and Virulence in *E. coli*

<p>As stated earlier in this chapter, another regulator, the virulence activator RfaH, has recently been linked to biofilm formation in *E. coli* (Beloin et al. 2006). A mutation in *rfaH* was shown to derepress biofilm formation in several *E. coli* strains, including uropathogenic strain 536. Since expression of several *E. coli* virulence-associated genes depends on RfaH, the increased biofilm phenotype of the nonvirulent *rfaH* mutant of strain 536 (Nagy et al. 2002) indicates that RfaH-dependent biofilm formation and virulence gene expression are mutually exclusive processes and that biofilm formation

may not be regarded as a virulence trait per se. This idea is currently reinforced by other studies also showing inverse regulation of virulence and biofilm-promoting factors in bacteria such as *Pseudomonas aeruginosa*, *Xanthomonas campestris*, and *Bordetella bronchiseptica* (Dow et al. 2003; Goodman et al. 2004; Irie et al. 2004; Kuchma et al. 2005). Recent data consistently indicate that biofilm of uropathogenic *E. coli* must be formed at the right place under appropriate conditions, and that this may also promote virulence under certain growth conditions (Anderson et al. 2003; Justice et al. 2004). These results suggest that biofilm formation definitely plays a role in the persistence of bacteria rather than being directly implicated in the infective mechanism itself. However, biofilms constitute reservoirs of bacteria that are potentially virulent, and the switch from biofilm to virulence and vice-versa might well be controlled by several regulators such as RfaH in *E. coli.*

<heading3>Influence of Other Global Regulators on *E. coli* Biofilm Formation

<p>Two regulators, H-NS and RpoS, associated with responses to environmental conditions, also play a role in modulating biofilm formation. H-NS is a nucleoid-associated protein that has been shown to regulate a large number of genes in *E. coli* (approximately 5% of the *E. coli* K-12 genome), including numerous cell envelope components such as flagella, type 1 fimbriae, LPS, and colanic acid, most of them linked to environmental stimuli including pH, oxygen, temperature, and osmolarity (Dorman and Bhriain 1992; Sledjeski and Gottesman 1995; Olsen et al. 1998; Soutourina et al. 1999; Hommais et al. 2001; Soutourina and Bertin 2003; Dorman 2004). The pleiotropic nature of the H-NS effect within the cells, as well as the fact that a mutation in the *hns* gene results in a reduction in the growth rate (Barth et al. 1995), is hardly compatible with a clear definition of its role in *E. coli* biofilm formation. Nevertheless, H-NS appears to be necessary for *E. coli* to attach to sand columns when it is grown under oxygen-limited conditions (Landini and Zehnder 2002). H-NS often interferes with the expression of genes that depend on the RpoS sigma factor. This interference occurs both by competing with RpoS for binding to the promoter of these genes and by indirectly repressing *rpoS* translation and stimulating RpoS turnover (Hengge-Aronis 1996, 2002). Whereas *rpoS* expression in *E. coli* seems unchanged between cells grown as a planktonic culture in a chemostat and as a biofilm (Adams and McLean 1999; Schembri et al. 2003b; Beloin et al. 2004), the role of RpoS in biofilm formation remains controversial. Depending on the experimental setup, an *rpoS* mutation has different effects on *E. coli* biofilm development, ranging from a strong negative effect to a positive effect (Adams and McLean 1999; Corona-Izquierdo

and Membrillo-Hernandez 2002; Jackson et al. 2002b; Schembri et al. 2003b). These results pinpoint the difficulty of comparing studies performed using different experimental protocols, and consequently definitive conclusions cannot be drawn concerning the role of RpoS in the formation of *E. coli* biofilms.

<heading1>Conclusions

<p>The study of bacterial surface colonization and biofilm formation represents a rapidly expanding field of investigation, and we are only now beginning to fathom the complexity of how bacteria adjust their lifestyle when confronted with surface contact and community growth.

<p>The amenability of *E. coli* to genetic analyses makes it a valuable biofilm experimental model applicable to a wide spectrum of molecular biology approaches, from classical to genome-wide genetic analyses. Indeed, the growth of *E. coli* under different static and dynamic biofilm experimental set-ups, combined with advanced microscopic analyses, have led not only to identification of a large repertoire of adhesins, but also to the uncovering of complex interplays between regulatory networks involved in biofilm lifestyle. Moreover, the progressive switch from the reliable and convenient laboratory workhorse *E. coli* K-12 to wild commensal and pathogenic isolates has revealed unsuspected physiological capacities and unexplored biological resources that can be used by *E. coli* species to operate within a biofilm.

<p>Therefore, in addition to the molecular details of its own biofilm formation, the use of the most extensively studied living organism thus far, *E. coli*, is likely to represent a coherent choice when venturing into the complex area of experimental molecular multispecies microbiology. Indeed, although bacterial flora exist primarily as multicellular biofilm communities, the absence of an appropriate approach has hampered the investigation of these issues in a complex mixed-biofilm context. We believe that some fundamental questions of the molecular intricacies of commensal–pathogen interactions may also be efficiently addressed in model *E. coli* mixed consortia. Hence, beyond their fundamental, clinical, and ecological relevance, *E. coli* species have probably not yet revealed all of their secrets, and might significantly contribute to bacterial biofilm research in the near future.

<acknowledgements>

<formalp><phead1>Acknowledgements</phead1><p>We thank Sandra Da Re, Jaione Valle and Benjamin Le Quéré for helpful suggestions and critical reading of the

manuscript, supported by the Ministère de l'Education Nationale, de l'Enseignement Supérieur et de la Recherche (MENESR) and by the Fondation pour la Recherche Médicale (FRM) C.B. and J.M.G. are supported by grants from the Institut Pasteur, the CNRS URA 2172, the Network of Excellence EuroPathoGenomics; LSHB-CT-2005-512061 and the Fondation BNP PARIBAS.
</acknowledgements>

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