



"It's a gut feeling" - *Escherichia coli* biofilm formation in the gastrointestinal tract environment

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Not all is shit - *Escherichia coli* biofilm formation with a focus on the gastrointestinal tract

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Abstract *Escherichia coli* is one of the most frequent commensals and pathogens of the human gastrointestinal tract. In this review, we discuss biofilm formation of commensal, probiotic and pathogenic strains of *E. coli*. Biofilm formation of *E. coli* is highly variable, although distinct regulatory pattern of *rdar* biofilm formation arise in certain pathovars. Interestingly, *E. coli* Nissle 1917 has its regulatory *rdar* biofilm expression pattern conserved for 100 years. Various environmental signals relevant for the gastrointestinal tract affect biofilm formation. Biofilm formation in the gastrointestinal tract has mainly been characterized in interaction with epithelial cells. Contribution of the vast number of core genome and pathovar specific proteinaceous surface appendages to biofilm formation has hardly been investigated.

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1 Introduction

The conventional view is that the natural habitat of the versatile species *Escherichia coli* is the intestine of humans and warm and cold-blooded animals, namely mammals, birds and reptiles. *E. coli* is one of the earliest colonizers of the gastrointestinal tract; although eventually, it is a minor component of the colonic gut microbiome in humans, where it represents less than 0.1% of the total bacterial cells (Eckburg et al., 2005). Nevertheless, due to the overall high cell density in the colon, this small percentage translates into a number around 10^8 cells/ml. Although generally defined as a commensal, *Escherichia coli* actually contributes to human physiology through the digestion of food and production of vitamin B and K with some strains even having probiotic features. On the other hand, *E. coli* is also one of the most prevalent human pathogens, causing various intestinal and extra-intestinal diseases, including being suspected to be the cause of sudden infant death syndrome (Bettelheim and Goldwater, 2015; Blount, 2015; Nataro and Kaper, 1998). Not surprisingly, due to its impact on human health, *E. coli* is one of the most well-investigated bacterial species, but nevertheless little is known about *E. coli* as there is a tremendous diversity within the species (Blount, 2015). Besides in its primary habitat, *E. coli* is also found in aquatic and terrestrial habitats as well as associated with plants (Meric et al., 2013). As a particular *E. coli* strain transmits through the gut in a time period varying from weeks to years, environmental and host associated strain populations are most probably tightly interconnected and shape each other.

The natural life style of microorganisms is considered to be biofilm formation, a tissue-like multicellular assembly with or without being associated with biotic or abiotic surfaces. The developmental process of biofilm formation conventionally initiates with the slow-down of the movement of a single motile cell in the liquid phase and restarts with the active or passive dispersion of cells from the mature three-dimensional biofilm with complex architecture. Adhesion factors and a variety of extracellular matrix components shape the development from the single attached cell over microcolony formation towards three-dimensional biofilms (Hobley et al., 2015). The predominance of this multicellular life style in nature has been attributed to survival advantages of multicellular assemblies compared to single cells such as tolerance to various forms of

stress including desiccation and tolerance against antimicrobial substances and the immune response of higher organisms.

E. coli is also capable to form biofilms. However, although biofilm formation is a well-defined process, the > 20% genome variability and the diversity of environmental niches suggest a hardly explored variability in biofilm formation within the different *E. coli* species. This review focuses mainly on biofilm formation of commensal, probiotic and pathogenic gastrointestinal *E. coli*. In the benign status, the microbiota, including *E. coli*, resides in the outer loose mucin layer, separated from the epithelial mono-cell barrier by an inner dense mucin barrier (Johansson et al., 2013). Upon infection and a disturbed immune status, pathogens penetrate and interact with the epithelial lining and beyond. Thereby, in some pathovars biofilm formation is integrally included in the virulence phenotype, while other pathovars only transiently form biofilms. Biofilm formation is thus tightly regulated against motility and virulence in response to gastrointestinal intrinsic signals.

2 Biofilm formation

2.1 Biofilm *in vitro* models

Model systems are needed to make biofilm formation molecularly investigable. The classical model species *Pseudomonas aeruginosa* has imprinted the view on mature biofilms as mushroom-like structures under the conditions of continuous flow (Klausen et al., 2003). A broad number of model systems exist to analyze biofilm formation of *E. coli*. A wide-spread popular system is the ‘so-called’ rdar (red, dry and rough) colony morphology, a distinct colony morphology type. This phenotype is indicative for the expression of biofilm extracellular matrix components curli fimbriae and cellulose concomitantly regulated by the major biofilm regulator CsgD (Fig. 1; (Hufnagel et al., 2015; Römling, 2005; Römling et al., 1998)). In a surprisingly high number of situations, structural and regulatory components of the ‘rdar’ biofilm contribute to biofilm formation, for example, in the colonization of plants and fruits, but also of tumor tissue (Pawar et al., 2014; Yaron and Römling, 2014). As ‘rdar’ is based on the binding of the azo-dye Congo Red semi-specific for amyloids and 1,4- and 1,3- beta glucans, colony morphology biofilms producing the exopolysaccharide poly- β -1,6-N-acetylglucosamine,

for example, can also be assessed. The rdar biofilm is the most intensively investigated biofilm with respect to structural components and regulation (see below ‘Signals leading to biofilm formation’). At the center of biofilm regulatory network, lies the CsgD protein, transcribed from the *csgDEFG* locus characterized by an eukaryotic-like promoter that includes a 117 bp long leader sequence with regulatory sequences far up- and downstream of the transcriptional start site (Holmqvist et al., 2013). Complex transcriptional to post-translational regulatory mechanisms grossly affect and fine-tune bistable CsgD expression (Grantcharova, 2010; Holmqvist et al., 2010; Mika and Hengge, 2013; Simm et al., 2014). Of note, the green tea polyphenol epigallocatechin gallate (EGCG) downregulates *csgD* expression via the sRNA RybB and interferes with curli assembly (Serra et al., 2016).

Another frequently applied model system is biofilm formation on an abiotic surface, such as glass or plastic walls of an incubation tube under static or shaken culture conditions. A ‘rdar’ biofilm (Fig. 1), but also biofilms with alternative extracellular matrix components and regulation have been identified in this conditions. Molecular analysis of biofilm formation of *E. coli* under continuous flow is scarce, thus the components that built up the biofilm under constant nutrient flow require to be further investigated (Reisner et al., 2006).

Although residing in the mucus in distance from the intestinal cell layer, *E. coli* often forms biofilms on the epithelial lining under pathophysiological conditions. Therefore, factors that affect biofilm formation can be conveniently investigated in cell culture models (Fig. 1). Intracellular biofilms, so called intracellular pods, as formed by uropathogenic *E. coli*, are not the subject of this review (Anderson et al., 2003).

2.2 Biofilm formation in the environment

Outside the human body, *E. coli* is thriving in oligotrophic water and soil (Gordon and Cowling, 2003; van Elsas et al., 2011), an inherent feature of the *E. coli* species (Na et al., 2006). These observations are consistent with early studies that suggest low temperature, stress and nutrient deprivation to favour expression of biofilm genes (White-Ziegler et al., 2008). In these environmental habitats, cross-kingdom biofilms are rather the rule than the exception and consist of bacteria, fungi, algae and/or protozoa

(Costerton et al., 1999; Watnick and Kolter, 2000). Biofilms containing *E. coli* have been detected in fresh water streams and drinking water sources (Ahmed et al., 2013; Moreira et al., 2012). Thus, *E. coli* might integrate into preformed interkingdom biofilms, which serves as a vehicle for pathogen transmission (Alam et al., 2007). In any case, the presence of biofilms in water has been linked to epidemic peaks of diarrhea (Ahmed et al., 2013). Also, food borne transmission of commensal and pathovar *E. coli* has its basis in the attachment and biofilm formation on fresh produce such as lettuce, tomatoes and rocket (Jeter and Matthyse, 2005; Richter et al., 2014; Yaron and Römling, 2014).

3 Pathovar specific biofilm formation

Biofilm formation in *Escherichia coli* is highly variable, not surprising probably, considering the high genome variability and the various ecological niches *E. coli* is thriving in. Initial multi locus enzyme electrophoresis, MLEE, analysis (Selander and Levin, 1980) and subsequent multi locus sequence typing MLST (Maiden et al., 2013) indicated that many *E. coli* pathovars arose independently more than once throughout the phylogenetic tree with ETEC arousal most readily observed (Chaudhuri and Henderson, 2012). A simple, but surprisingly discriminative triplex PCR approach divided *E. coli* into five major phylogroups A, B1, B2, D, E, and less frequent C and F groups, still widely used until today (Clermont et al., 2013; Tenaillon et al., 2010). Subsequently, though, this classification is replaced by a next generation sequencing (NGS) based classification scheme with currently more than 2500 *E. coli* genomes available, with phylogrouping being congruent with genome comparison (Chaudhuri and Henderson, 2012). Currently the *E. coli* pangenome is estimated to be more than 16.000 genes, while the shared core genome is, with less than 1.500 genes, remarkably small (Rouli et al., 2015; Touchon et al., 2009). A significant portion of the accessory genome codes for virulence factors and metabolic genes and shapes the adaptation to various niches including the human gastrointestinal tract (Leimbach et al., 2013). Thus, it is not unexpected that genome variability grossly affects biofilm formation. On the other hand, even single nucleotide polymorphisms have been demonstrated to alter biofilm physiology dramatically (Römling et al., 1998).

3.1 Biofilm formation by commensal *E. coli*

Commensal *E. coli* isolates show the largest possible range of biofilm regulatory patterns with respect to 'rdar' biofilm formation on agar plates (Bokranz et al., 2005) ranging from a no rdar morphotype to a semi-constitutive rdar morphotype expressed at both 28°C and 37°C. In addition, expression variants include curli fimbriae or cellulose only (Fig. 1). The full biological impact of rdar morphotype variability is mostly unknown, although absence of rdar morphotype expression is associated with the invasive phenotype of *Shigella* and enteroinvasive *E. coli*. In general, bacterial cells expressing individual extracellular matrix components curli and cellulose, interact in a distinct and even opposite way with intestinal epithelial cells with respect to adhesion, invasion as well as induction of proinflammatory cytokine production (Monteiro et al., 2009; Saldaña et al., 2009; Wang et al., 2006). As a general pattern curli fimbriae stimulate these phenotypes, which are inhibited upon co-expression of cellulose. In the colon, *E. coli* resides in the outer mucin layer, separated from the epithelial mono-cell barrier by the tighter inner mucin layer (Johansson et al., 2013). Indeed, the sheer presence of mucin stimulates biofilm formation (Bollinger et al., 2006). Commensal *E. coli* growing in the mucus cannot use this nutrient source, but utilize mono-, disaccharides and other simple molecules derived from complex glycoprotein degradation by tightly interacting obligate anaerobes (Conway and Cohen, 2015; Ng et al., 2013). How nutrient provision in the gut affects biofilm formation is largely unknown, but, for example, monosaccharide availability and the PTS system have been shown to affect biofilm formation in various bacteria (Houot et al., 2010; Sutrina et al., 2007). In addition, secreted immunoglobulin A favors biofilm development of *E. coli* in the intestinal mucosa in a type 1 fimbriae-dependent manner (Bollinger et al., 2006). In total, the commensal model organism *Escherichia coli* K-12 has seven additional chaperone usher pathway fimbriae in addition to type 1 fimbriae (Korea et al., 2010), which have the potential to mediate biofilm formation.

3.2 Pathogenic *E. coli* have acquired different abilities to form biofilms

E. coli has evolved and adapted to a pathogenic lifestyle through the acquisition of virulence genes that confer advantages to colonize the host environment (Kaper et al.,

2004; Leimbach et al., 2013). Phenotypically pathogenic *E. coli* are divided into two groups, intestinal *E. coli* (InPEC) and extraintestinal *E. coli* (ExPEC). The differences in the pathogenic potential is reflected in the phylogeny as *E. coli* of phylogroup A, B1, C and E are commonly associated with intestinal disease, while *E. coli* of phylogroups B2, D and F are predominantly associated with extraintestinal diseases (Fig. 3), indicating that a specific genetic background (*i.e.* phylogroup) provides pathogenic advances in distinct host environments. However, several exemptions from this general association of phylogroups to pathotypes exist indicating parallel evolution of *E. coli* pathotypes on multiple occasions and ready arise of certain pathovars in the *E. coli* population. (Bielecki et al., 2014; Chaudhuri and Henderson, 2012; Hazen et al., 2013; Reid et al., 2000; von Mentzer et al., 2014).

The InPEC group includes pathogens that infect and colonize the gastrointestinal tract and typically cause secretory or bloody diarrhea. The InPEC includes enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), Shiga toxin (STEC) producing *E. coli* that includes enterohemorrhagic (EHEC), enteroinvasive *E. coli* (EIEC) and *Shigella* spp., adherent-invasive *E. coli* (AIEC) and diffusely adherent *E. coli* (DAEC) (Kaper et al., 2004; Leimbach et al., 2013). While some pathovars affect the small intestine, where the mucus in the small bowel is unattached to the epithelium, others cause infection in the large intestine (Fig. 2 and 3; (Johansson et al., 2013)). Extraintestinal *E. coli* (ExPEC) includes uropathogenic *E. coli* (UPEC) and newborn meningitis *E. coli* (NMEC) and mainly belongs to the B2 and D phylogroups. The molecular mechanisms behind the pathogenicity of the *E. coli* pathovars have been reviewed extensively (Croxen and Finlay, 2010; Croxen et al., 2013; Kaper et al., 2004; Nataro and Kaper, 1998).

The diversity of acquired pathogenicity islands/islet/genes in the pathovars including virulence factors and the unique chromosomal background of different *E. coli* pathotypes suggest that *E. coli* pathovars have unique regulatory and structural capacities to form biofilms. For examples, pathogenic *E. coli* express a wide array of pathovar specific fimbriae. On the other hand, though, there are biofilm components on the core genome of *E. coli* belonging to the 1500 common genes. Well-investigated amyloid curli fimbriae are involved in late stage biofilm formation (Farfan and Torres, 2012; Monteiro et al.,

2012). Curli expression, which, according to current knowledge is synonymous with expression of the major biofilm regulator CsgD, varies widely in expression pattern and is strain and/or pathovar dependent. For instance, sepsis and UTI (urinary tract infection) isolates frequently express curli fimbriae also at 37 °C (Bian et al., 2000), while in EPEC, ETEC and ETEC curli are often only expressed at ambient temperature (Szabó et al., 2005; Uhlich et al., 2001). In EIEC and *Shigella* spp., curli and *csgD* expression is often inactivated (Sakellaris et al., 2000).

Also type 1 fimbriae are expressed by the majority of *E. coli*, but their role in biofilm formation can differ between commensal and pathogenic *E. coli*. For instance, type 1 fimbriae are important for biofilm in the laboratory K-12 strain, but not in EHEC O157:H7 (Beloin et al., 2004; Roe et al., 2001). Interestingly, different *fimH* alleles confer different tissue tropism with respect to adhesion to epithelial cells via binding to mono-mannose receptors, affecting the ability of the bacteria to attach and invade epithelial cells (Khan et al., 2007; Martinez et al., 2000; Weissman et al., 2006). Similarly, during biofilm formation, different *fimH* alleles can result in different functionality and role of type 1 fimbriae. Finally, type 1 fimbriae exhibit a unique catch bond adhesion mechanism, which provides stronger attachment to the surface when tensile force is applied (Aprikian et al., 2011), which might be the initiation phase of biofilm formation.

Autotransporter adhesins are members of the type V secretion system and associated with autoaggregation and biofilms. Antigen 43 (Ag43) promotes cell-to-cell adhesion and aggregation at the initial stages of biofilm formation. Ag43 is present at higher frequency in non-O157 EHEC strains compared to O157:H7 (Verstraete et al., 2013).

Cellulose, a polysaccharidic component of the biofilm extracellular matrix, is frequently co-expressed with curli fibres, but can also be regulated independently (Beloin et al., 2008; Bokranz et al., 2005; Monteiro et al., 2009). Colanic acid is another core genome encoded exopolysaccharide. Although reported to promote biofilm formation, its contribution to this process, though, is not well defined (Matthysse et al., 2008; May and Okabe, 2008). The common exopolysaccharide poly-*N*-acetylglucosamine (PNAG) is expressed in *E. coli* K-12 upon deletion of the global carbon storage regulator CsrA, which represses translation of the *pga* operon. Although PNAG is, as cellulose, an

exopolysaccharide commonly produced in biofilms from diverse bacterial species, the *pga* operon is not present in all *E. coli* strains (Cerca et al., 2007).

Below we discuss the pathovar specific biofilm components. Besides investigated for biofilm formation *in vitro*, frequently, those surface components have been characterized in interaction with epithelial cells and gut colonization.

3.3 ETEC specific biofilm mediators

ETEC infections is a major cause of dehydrating diarrheal disease primarily affecting children less than five years of age and travellers to low- and middle-income countries with expression of the heat labile toxin (LT) and the heat stable toxin (ST) as hallmark (Qadri et al., 2005). In addition, ETEC harbours distinct surface proteins, more than 27 identified and proposed colonization factors (CFs) and adhesins, which promote colonization of the small intestinal epithelium (Gaastra and Svennerholm, 1996). Biofilm production and adherence are associated with CFs and multiple CF genes located on the plasmid as well as the chromosome has been implicated to participate in biofilm development. For example, ETEC strains expressing the colonization factors CFA/I, CS1, CS2 or CS3 were reported to produce higher levels of biofilm than non-piliated ETEC strains (Liaqat and Sakellaris, 2012).

The toxigenic invasion loci A (Tia) and toxigenic invasion loci B (*tibAB*) (Elsinghorst and Weitz, 1994; Fleckenstein et al., 1996) are involved in adhesion and biofilm formation (Sherlock et al., 2005). The expression of TibA and Tia adhesins by 8% and 10% of ETEC strains is associated with certain colonization factor profiles (Sjöling et al., 2015). The presence of type IV pilus CS2, longus (Giron et al., 1994), is predominantly associated with expressing of CS1+CS3, CS2+CS3 and CFA/I colonization factors (von Mentzer et al., 2014) and associated with biofilm formation (Cruz-Cordova et al., 2014). ETEC isolates might vary considerably in their ability to form biofilms in the host and in the environment with consequences for dissemination and virulence properties. However, a systematic analysis of the biofilm formation capacity of ETEC strains has not been performed.

3.4 Biofilm formation by enterohemorrhagic *E. coli*

Enterohemorrhagic *E. coli* (EHEC) belong to the Shiga toxin-producing *E. coli* (STEC) where biofilm formation has been recently reviewed (Vogeleer et al., 2014). EHEC colonize both the distal ileum as well as of the colon. EHEC attaches to the epithelial cells via the use of pili, *E. coli* common pilus ECP and hemorrhagic pilus, HCP (Xicohtencatl-Cortes et al., 2009), as well as mucin binding flagella (Nagy et al., 2015; Xicohtencatl-Cortes et al., 2009). Initial attachment to and biofilm formation on spinach leaves and stainless steel surfaces is mediated by curli fimbriae in EHEC outbreak strains (Carter et al., 2016). Further, a multitude of adhesins are expressed in STEC (Klapproth et al., 2000; Nicholls et al., 2000), among them EspA, promoting attachment (Ebel et al., 1998; Sharma et al., 2016). Somewhat surprising, enterohemorrhagic *E. coli*, EHEC, strains show a characteristic pathovar dependent loss of rdar biofilm formation. Indeed, many strains have a disrupted or downregulated *mlrA* gene, which is required to transcriptionally elevate expression of the determinative biofilm regulator *csgD* (see below). Impaired *mlrA* activity is due to a *stx1* bearing phage insertion and/or abolished or attenuated RpoS activity. The latter finding is surprising as the stationary phase sigma factor RpoS is considered a major stress resistance determinant. Consequently, *E. coli* serotype O157:H7 strains usually do not display the rdar morphotype (Uhlich et al., 2013). Upon excision of the *stx1* prophage, though, rdar biofilm formation is restored (Uhlich et al., 2016).

3.5 EPEC biofilm formation

Enteropathogenic *E. coli* (EPEC) belong to the STEC type together with EHEC divided into typical EPEC expressing the bundle-forming pili (Bfp) and atypical EPEC (aEPEC) that do not express Bfp. Typical EPEC forms microcolonies, also known as localized adherence (LA), on epithelial cells in culture in a Bfp-dependent way (Bieber et al. 1998), while aEPEC show a looser LA-like adherence. Biofilm formation in EPEC is variable and depends on type 1 fimbriae, Ag43, curli and cyclic di-GMP (Moreira et al., 2006; Weiss-Muzkat et al., 2010; Nascimento et al., 2014). In addition, a secreted lipoprotein SsIE mediates biofilm formation in EPEC (Baldi et al., 2012), encoded also by several other *E. coli* pathotypes and commensal *E. coli* (Moriel et al., 2012; Decanio et al., 2012; Luo et al., 2014; Sjöling et al., 2015).

Albeit generally low, adherence and pellicle formation in standing culture was more prevalent in aEPEC strains from patients compared to asymptomatic controls (Nascimento et al., 2014). The biofilm behaviour including rdar of aEPEC strains is generally mostly observed at 26°C (Weiss-Muzkat et al., 2010; Nascimento et al., 2014).

3.6 DAEC

DAEC are characterised by a diffuse pattern of adherence with little aggregation on epithelial cells (Fig. 2). The fimbrial adhesin F1845 (Bilge et al., 1989), Dr fimbrial, and afimbrial adhesins (Afa) bind to the brush-border associated receptors leading to brush border lesions and high adherence. The characteristics of DAEC have been extensively reviewed (Le Bouguenec and Servin, 2006; Servin, 2005, 2014).

3.7 EAEC

Enteroaggregative *E. coli* (EAEC) colonize the small and large intestine (Hebbelstrup Jensen et al., 2014), but can also cause extraintestinal infection. The single-layer aggregative adherence pattern on epithelial cells of the gut characteristic for this pathotype is referred to as “stacked-brick” configuration (Nataro et al., 1987). Major interbacterial and host cell adhesion components are pathovar specific aggregative adherence fimbriae (AAF), which belong to the Dr adhesins family (Czczulin et al., 1997; Nataro et al., 1992) and afimbrial adhesins (reviewed in (Estrada-Garcia and Navarro-Garcia, 2012)), type 1 fimbriae and other components such as the secreted small 10-kDa protein dispersin. This small protein was shown to lead to dispersion of the cells along the intestinal epithelium (Sheikh et al., 2002) by shielding the negative charge of the bacterial outer membrane, allowing extension of the positively charged AAFs from the surface (Velarde et al., 2007). Biofilm formation has been suggested to be a major transmission and virulence factor as out-break strains on several occasions have been found to display enhanced biofilm formation including the EAEC/EHEC O104:H4 hybrid strain related to the recent major outbreak (Hebbelstrup Jensen et al., 2014; Karch et al., 2012; Kong et al., 2015).

3.8 Crohn’s disease associated *E. coli* biofilm formation

In healthy human beings, mucus layers separate the epithelial lining from the gut microbiome. In Crohn's disease (CD), adherent-invasive *E. coli* (AIEC) with specific features are found in close association with epithelial cells (Darfeuille-Michaud et al., 2004). AIEC attachment is facilitated by type 1 fimbriae binding to specific host receptors upregulated in patients with CD (Barnich et al., 2007; Boudeau et al., 2001; Carvalho et al., 2009) and supported by flagella (Barnich et al., 2003). Further, adhesion is mediated by interaction of the bacterial chitinase ChiA with the host chitinase 3-like-1 (CHI3L1) via chitin binding domains (Low et al., 2013; Mizoguchi, 2006). Flagella and type 1 fimbriae are co-regulated in the Crohn's disease isolate *E. coli* strain LF82 with type 1 fimbriae production partially activated through downregulation of the second messenger cyclic di-GMP (Claret et al., 2007). The extracellular matrix component cellulose triggers induction of colitis in IL-10 deleted mice, which are prone to inflammation (Ellermann et al., 2015).

3.9 Biofilm formation of Nissle 1917

Escherichia coli Nissle 1917 is one of several established probiotic *E. coli* strains, isolated in 1917 by the German physician Nissle. *E. coli* Nissle 1917 expresses a particular rdar biofilm feature that distinguishes it from other *E. coli* strains with CsgD and di-guanylate cyclase YedQ independent cellulose expression uncoupled from ambient temperature expressed curli (Monteiro et al., 2009). This regulatory pattern is surprisingly stable as *E. coli* Nissle 1917 clonal strains isolated almost 100 years later from pigs show similar regulation (Kleta et al., 2006; Monteiro et al., 2009). The ecological impact of this regulation is not clear though. A screen of different fimbriae identified F1C fimbriae as being required for biofilm formation at 37°C on abiotic surfaces and intestinal colonization (Lasaro et al., 2009). Outcompetition of pathogenic *E. coli* strains and other species during biofilm formation might contribute to the probiotic features of Nissle 1917 (Hancock et al., 2010; Jiang et al., 2014; Lasaro et al., 2009; Rund et al., 2013).

4 Signals leading to biofilm formation

4.1 Physiological and environmental signals linked to biofilm found in the

gastrointestinal (GI) tract

Biofilm formation represents a very energy-costly process for bacterial cells, due to the production of the extrapolymeric substance and adhesion factors as described above; on the other hand, biofilms show increased resistance to environmental stresses and to the host immune system, as well as tolerance to antibiotics. In order to balance the pros and cons of the biofilm mode and transition to the planktonic life style, bacterial cells integrate diverse intra- and extracellular signals with a previously unprecedentedly observed complexity. Signals directly sensed are linked to the bacterial population density and composition, the presence of specific nutrients, light etc.

In the GI tract, commensal and pathogenic *E. coli* face a wide range of environmental signals (*e.g.*, temperature, oxygen, nutrients), as well as signals from the host (*e.g.*; bile salts, taurine, neuroendocrine hormones like norepinephrine and dopamine) and from other commensal bacteria (*e.g.*, quorum sensing (QS) signals, indole, polyamines). In this section, we will focus on a selection of environmental, host- and bacteria-related signals that *E. coli* can perceive in the GI tract and will describe how they regulate biofilm determinants. Some of these physiological and environmental signals are linked to second messenger signaling by modified nucleotides, such as cyclic-di-GMP (c-di-GMP), possibly the main effector for biofilm formation and maintenance in *E. coli*. The reader can find detailed descriptions of the mechanisms of c-di-GMP signaling in a number of extensive review articles recently published (Hengge, 2009; Jenal and Malone, 2006; Römling et al., 2013; Sondermann et al., 2012).

Quorum sensing

Quorum sensing molecules, also known as autoinducers (AI), are one of the most studied signals affecting biofilm formation in a broad range of bacteria. So far, several AI have been characterized that cluster in three classes: AI-1, AI-2, AI-3. Each signaling system relies on a specific autoinducer synthase and a cognate receptor. AI-1 molecules are synthesized by so called LuxI-type proteins and belong to the molecular class of acyl homoserine lactones (AHLs) showing a conserved homoserine lactone ring connected through an amide bond to a variable acyl chain (reviewed in (Reading et al.,

2009)). The acyl chains vary considerably in length (from 4 to 18 carbon atoms) and in chemical modifications: for instance, the third carbon of the chain is often derivatised to a carbonyl group, a hydroxyl group or a methylene moiety (Marketon et al., 2002; Whitehead et al., 2001). Such chemical variety confers these molecules properties of either intraspecies or interspecies signals: indeed, some AHLs are species-specific, while others are able to induce responses in a broader range of bacteria, mostly Gram-negative. The ability of AHL to act as signal molecules is mediated by their recognition by specific receptor proteins belonging to the LuxR family.

Unlike most Gram-negative bacteria, *E. coli* genome seems to lack LuxI synthases, and it does not produce AI-1. However, it possesses a LuxR homolog, SdiA (for Suppressor of cell Division Inhibitor (Wang et al., 1991)). SdiA can respond to AHLs secreted by other bacterial species (Dyszal et al., 2010a; Hughes et al., 2010; Lee et al., 2007; Smith et al., 2008; Soares and Ahmer, 2011) and even to synthetic AHLs (C6-HSL and oxo-C6-HSL) (Van Houdt et al., 2006). Thus, it appears that *E. coli* can sense AI-1 produced by other bacteria, probably as a mechanism to monitor their presence, but does not produce any own AHL. The precise role of SdiA, and how this regulator relays sensing of AI-1 from other bacterial species to gene expression, still remain elusive, and its involvement in *E. coli* biofilm formation is controversial. For instance, both deletion and overexpression of the *sdiA* gene result in inhibition of curli fibers production via transcriptional repression of the curli operons (Lee et al., 2009; Lee et al., 2008). The SdiA proteins can bind several ligands in addition to AI-1, including 1-octanoyl-*rac*-glycerol (OCL) (Nguyen et al., 2015), an ubiquitous monoacylglycerol, present in both eukaryotes and prokaryotes (Alvarez and Steinbüchel, 2002), and the sugar xylose (Yao et al., 2007; Yao et al., 2006), and can thus respond to different environmental and physiological cues. Likewise, the transcription of the *sdiA* gene is under the control of up to 15 regulators, again suggesting a very finely tuned control of intracellular SdiA concentrations in response to environmental conditions. Thus, it is likely that SdiA can integrate multiple signals, possibly having even opposite effects on gene expression depending on the conditions encountered by the bacterium. The most direct effect of SdiA on the transition between sessile and planktonic lifestyles is the repression of cellular motility, reported by several groups (Dyszal et al., 2010b;

Kanamaru et al., 2000; Lee et al., 2007; Lee et al., 2008), possibly via modulation of c-di-GMP signaling. Indeed, inactivation of SdiA affects the expression of YhjH, a c-di-GMP phosphodiesterase involved in regulation of curli fibres and flagella (Lindenberg et al., 2013; Pesavento et al., 2008), thus suggesting an effect of SdiA on the pivotal second messenger c-di-GMP.

Autoinducer-2 (AI-2) is widely conserved in both Gram-negative and Gram-positive bacteria and has therefore been proposed as a “universal” bacterial signal able to mediate both inter- and intraspecies communication (Surette et al., 1999). AI-2 is a furanosyl borate diester and one of the few biological molecules known to have a boron atom as a constituent (reviewed in (Pereira et al., 2012b)); it is synthesized by the LuxS protein starting from S-ribosylhomocysteine (SRH) as a non-toxic product of S-adenosylmethionine (SAM) metabolism (Pereira et al., 2012b; Schauder et al., 2001; Xavier and Bassler, 2003). In contrast to AI-1, *E. coli* possesses all the required machinery for synthesis and detection of AI-2. AI-2 synthesized by non-pathogenic *E. coli* strains seems to be perceived by colon cells inducing an inflammatory response that is quickly dampened after few hours, thus suggesting that AI-2 can also serve as an interkingdom signal (Zargar et al., 2015). Three different classes of receptors for AI-2 have been described, which belong to the LuxP, LsrB or RbsB families (Armbruster et al., 2011; Chen et al., 2002; Miller et al., 2004). AI-2 is synthesized by LuxS, and actively secreted into the extracellular environment throughout cell growth. AI-2 is not able to passively diffuse through the membrane (Zhu and Pei, 2008); initial uptake of a reduced amount of AI-2 takes place through the phosphoenolpyruvate phosphotransferase system (PTS) probably without substrate phosphorylation. The small pool of intracellular AI-2 obtained through uptake by PTS is then phosphorylated (P-AI-2) by basal level of the LsrK protein (AI-2 kinase), relieving LsrR repression on *lsr* operon and initiating Lsr-dependent, transport and depletion of AI-2 (Pereira et al., 2012a). Altogether, the genes responding to AI-2 are organized in two divergent operons, *lsrACDBFG*, which comprises genes encoding an AI-2-specific uptake system and two enzymes (LsrF and LsrG) involved in AI-2 degradation and recycling, and the regulatory *lsrRK* operon. The LsrK protein phosphorylates AI-2, which, in this form,

binds the LsrR protein relieving its repression of the *lsrACDBFG* operon. Thus, when the AI-2 signaling system is activated, the signal molecule is promptly removed from the environment, preventing other bacteria to exploit the signal to regulate their behaviors (Roy et al., 2010; Xavier and Bassler, 2005a). As a consequence, and in contrast to other QS systems, AI-2 concentration in the culture medium of strains harboring the *lsr* system peaks during late exponential phase and quickly declines during stationary phase (Xavier and Bassler, 2005b).

By way of its ability to sequester and degrade AI-2 present in the environment, *E. coli* probably interferes with AI-2 communication of other bacterial species. This strategy can represent a huge advantage in complex niches: indeed, AI-2 quenching seems to be a conserved strategy evolved in enteric bacteria in order to control colonization of GI tract by other bacteria, including pathogenic species (Hsiao et al., 2014; Xavier and Bassler, 2005a). For example, by quickly consuming AI-2, *E. coli* can potentially modulate virulence factor production, motility, cellular adhesion dynamically modifying persistence, pathogenicity and dissemination of bacteria such as *Vibrio cholerae* and *Salmonella* Typhimurium (reviewed in (Pereira et al., 2012b)).

In *E. coli*, AI-2 stimulates the expression of a large set of biofilm-related genes (González Barrios et al., 2006; Herzberg et al., 2006; Li et al., 2007; Ren et al., 2004; Sperandio et al., 2001), including cellular chemotaxis proteins, flagella (Domka et al., 2006), extracellular polysaccharides (colanic acid (Prigent-Combaret et al., 2000), poly-*N*-acetylglucosamine (Wang et al., 2004)), adhesins (type I fimbriae (Pratt and Kolter, 1998), antigen 43 (Klemm et al., 2004), curli fibers (Prigent-Combaret et al., 2000)), and proteins involved in c-di-GMP processing (YhjH (Lindenberg et al., 2013; Pesavento et al., 2008)). Despite such variety of effects, biofilm stimulation by AI-2 might mainly depend on the regulation of flagellar activity (González Barrios et al., 2006; Herzberg et al., 2006; Ren et al., 2004). Initially, it was suggested that AI-2 flagellar activation might depend on QseBC two-component regulatory system, in which the phosphorylated QseB response regulator can directly bind the *flhDC* promoter, in turn stimulating the expression of the flagellar activator FlhDC (Sperandio et al., 2002). Although the sensor protein QseC does not respond directly to AI-2, but rather to other signals originating from other bacteria or from the host ((Sperandio et al.,

2003); see also next section), data still support the notion that the QseB response regulator is required to relay AI-2 sensing to motility (González Barrios et al., 2006), thus suggesting a cross talk between AI-2 and other signaling systems. Through control of FlhDC expression, AI-2 can impact cell physiology and the motile/sessile cell switch, as FlhDC also regulates FliZ, a protein able to counteract the activity of the alternative sigma factor RpoS (Pesavento et al., 2008; Pesavento and Hengge, 2012), and of YhjH, a c-di-GMP phosphodiesterase promoting cellular motility while inhibiting curli fibers production (Lindenberg et al., 2013; Pesavento et al., 2008). QseBC expression also requires the activity of another component, MqsR, as mutants in the *mqsR* gene fail to regulate flagella and cellular motility in response to AI-2 in a QseBC-dependent manner (González Barrios et al., 2006). MsqR belongs to the MsqR/MsqA (*ygiU/ygiT*) toxin-antitoxin system, in which the MsqR toxin is a mRNA interferase that cleaves target RNAs at GC(A/U) sites, while MsqA is its cognate antitoxin (Christensen-Dalsgaard et al., 2010; Yamaguchi et al., 2009). In addition to inhibiting MqsR endoribonuclease activity, MsqA is able to bind DNA and to negatively regulate expression of its own operon; MsqA inhibition on its own promoter seems to be enhanced when the toxin/anti-toxin is formed (Yamaguchi et al., 2009). *In vitro* binding assays showed that MqsR does not interact directly with QseBC mRNA, raising the possibility that its effect could be indirect (Yamaguchi et al., 2009). Although TA systems are clearly involved in biofilm formation, as well as being crucial in many other cell processes, such as persistence (reviewed in (Wen et al., 2014)), they are often strictly interconnected with other signaling systems, and their effects may strongly be influenced by the genetic backgrounds and by the experimental conditions used (Kasari et al., 2010). It is interesting to note that most data on AI-2 signaling in *E. coli* are based on microarray or transcriptomics data performed comparing mutants in the AI-2 signaling system to their wild type. These experiments, despite providing a global picture of gene expression, fall short in the identification of direct targets for LsrR, the only specific responder to AI-2 known until now (Taga et al., 2001). So far, only one work carried out in *S. Typhimurium* using ChIP (Chromatin immunoprecipitation)-chip analysis has attempted to specifically identify LsrR regulon. The results of this work indicated that LsrR can directly bind the *lsr* operons, as expected; however, no other

LsrR-bound promoter was found (Thijs et al., 2010). It is conceivable that LsrR is not the only regulator directly responding to AI-2, and other unknown receptors can sense the signaling molecule, and relay it to biofilm formation.

Autoinducer 3/epinephrine (epi)/norepinephrine (NE)

Both commensal and pathogenic strains of *E. coli* are able to synthesize a third quorum-sensing molecule independent of LuxS activity but whose production can be affected by *luxS* disruption (Sperandio et al., 2003; Walters et al., 2006). This molecule, named AI-3, is probably an aromatic amine, although its precise chemical structure has not been identified yet. AI-3 might resemble the mammalian hormones epinephrine and norepinephrine, as these molecules can interact with some components of the AI-3 quorum-sensing system (Sperandio et al., 2003). AI-3 signaling is conserved in several species that inhabit the human GI tract, including pathogenic and commensal strains (Clarke and Sperandio, 2005), and thus suggesting that this molecule represents an important signal for bacteria in the gut. AI-3 was firstly described as crucial player in pathogenesis of enterohemorrhagic *Escherichia coli* O157:H7 (EHEC), where it controls the expression of different virulence loci (Sperandio et al., 2003). This molecule is sensed by the QseC membrane histidine kinase, also potentially involved in AI-2 sensing, that undergoes autophosphorylation upon binding to AI-3. The cross-regulation between AI-2 and AI-3 sensing, as well as the signal transduction mechanisms coupling these signal molecules to regulation of gene expression, appear to be very intricate. For instance, although QseC forms a two component regulatory system with QseB, this response regulator can be present in a phosphorylated form even in the absence of the QseC sensor, suggesting cross talk between QseB and other sensor kinase(s) (Kostakioti et al., 2009). It is interesting that lack of QseB impairs AI-2-dependent expression of the *motA* gene, while the effect of QseC was not investigated (González Barrios et al., 2006). It would be important to understand whether, and how, AI-2 and AI-3 sensing can converge on QseB controlling its phosphorylation state in response to different signals. As already mentioned, QseB can control biofilm formation mainly through the modulation of FlhDC regulon (Clarke and Sperandio, 2005; González Barrios et al., 2006; Sperandio et al., 2002; Yang et al., 2014). However, in

pathogenic *E. coli* strains, QseB can repress the production of different structures involved in cell adhesion, including the *espA* gene, type 1 fimbriae (*fim*) and curli (Kostakioti et al., 2009; Sharma and Casey, 2014), thus impacting cell ability to colonize gut epithelia (Sharma and Casey, 2014). Another mechanism involving only a portion of the QseBC system is curli production, as it has been shown that QseC, but not QseB, affects regulation of curli genes (Sharma and Casey, 2014). It must be stressed that adaptation to a commensal vs. pathogenic lifestyle might have altered quite considerably the response of different *E. coli* strains to quorum sensing signals such as AI-2 and AI-3.

In addition to sensing the AI-3 signal, QseC is a bacterial adrenergic receptor, as it is able to bind and modulate its cognate response regulator in response to epinephrine and norepinephrine, two mammalian hormones present in the bloodstream and in the mucosa of the gastrointestinal tract, respectively (Lyte et al., 2011). Thus, in addition to AI-3, the QseBC system allows the bacteria to integrate these host signals with the control of biofilm determinants, contributing to bacterial community formation (Kostakioti et al., 2009; Sharma and Casey, 2014; Yang et al., 2014). It might be tempting to speculate that, as epinephrine and norepinephrine are able to trigger a sensor for AI-3, likewise this bacterial molecule might interact with the host's adrenergic receptors, thus representing an interkingdom signaling molecule. To our knowledge, however, no effects of AI-3 on eukaryotic cells are reported in literature yet.

Epinephrine/Norepinephrine sensing is not an exclusive feature of QseC histidine kinase, as QseEF, a secondary two-component regulatory system whose transcription depends on QseBC itself, was shown to respond to the two mammalian hormones in EHEC controlling strain virulence. Interestingly, QseEF does not sense bacterial AI-3 and is activated by other molecules such as sulfate and phosphate (Reading et al., 2009). QseEF is also conserved in non-pathogenic strains, thus controlling stress-related genes or adhesive structures could be its role in commensal bacteria.

Indole

Indole is an intermediate metabolic product of both biosynthesis and degradation of

the amino acid tryptophan. In tryptophan degradation, indole is produced by the enzyme tryptophanase (TnaA) together with ammonia and pyruvate, which are used as a source of carbon and nitrogen under starvation conditions (Newton and Snell, 1964). Although some reports suggested its active uptake by the protein complexes Mtr and AcrEF-TolC (Kawamura-Sato et al., 1999; Yanofsky et al., 1991), recent evidence indicates that indole can freely traverse the cell membrane (Piñero-Fernandez et al., 2011). Indole is produced by a large number of Gram-positive and Gram-negative bacteria, including *Escherichia coli*, and it might act as both an intra- and interspecies signal (reviewed in (Thijs et al., 2010)). A concentration ranging between 0.25 mM and 1.2 mM indole is found in human faeces; thus, it is likely that similar concentrations of this molecule are present in the GI tract, suggesting active degradation of tryptophan to indole by the bacterial gut flora. In addition to bacterial signaling, indole was shown to be an interkingdom molecule able to affect gene expression in epithelial cells (Bansal et al., 2010), mammalian cell metabolism and secretion of the incretin peptide GLP-1 (Chimerel et al., 2014), probably altering the lipid membrane potential (Chimerel et al., 2013; Wikoff et al., 2009).

In *E. coli*, the tryptophanase-encoding *tnaA* gene is under the control of the stationary phase sigma factor RpoS (Lacour and Landini, 2004); consistent with *tnaA* regulation by *rpoS*, indole can be detected in culture supernatants as cells approach stationary phase (Chant and Summers, 2007; Li and Young, 2013). Indole impacts a large number of cell processes, including transition to stationary phase (Lelong et al., 2007), plasmid stability (Chant and Summers, 2007), virulence factor in enterohemorrhagic *E. coli* (Hirakawa et al., 2009), biofilm formation (Domka et al., 2006; Lee et al., 2007; Martino et al., 2003; Wang et al., 2001). It has been reported that, at the onset of stationary phase, indole can reach a particularly high intracellular concentration (up to 60 mM), in comparison to a 4-5mM concentration in culture supernatants. This accumulation is only transient (20 minutes), but it appears to be crucial for bacterial survival in stationary phase (Gaimster et al., 2014). Finally, indole can work as an ionophore, controlling cell division through the modulation of membrane potential (Chimerel et al., 2012).

Early reports suggested that, in *E. coli*, indole can have a stimulatory effect on

biofilm formation as *tnaA* inactivation reduces biofilm formation and addition of the molecule restores the wild type (Martino et al., 2003). In contrast, however, later works suggested that indole represses biofilm development (Domka et al., 2006; Lee et al., 2007). As for other signal molecules, these discrepancies could be explained by the different conditions (e.g., presence or absence of glucose, a repressor of TnaA activity) and to the different *E. coli* strains used in these studies (Thijs et al., 2010).

Indole seems to have greater effects at 30°C than at 37°C; at lower temperatures its effect might be mediated by the AHLs sensor SdiA (see previous section), whose transcription is itself stimulated by indole (Lee et al., 2007; Lee et al., 2008). Indole can interfere with AHLs sensing by SdiA, as increasing concentrations of this molecule reduce transcription of SdiA-controlled gene *gadW* (Sabag-Daigle et al., 2012). However, indole also affects biofilm formation in a SdiA-independent fashion, as its inhibitory effect can be achieved both in wild type and *sdiA* mutant (Sabag-Daigle et al., 2012). Interestingly, indole production via tryptophanase is not very widespread in bacteria, and indole synthesis by *E. coli* can inhibit *P. aeruginosa* quorum sensing system increasing its competitiveness in mixed cultures (Chu et al., 2012). It is tempting to speculate that indole can be a *bona fide* QS molecule in *E. coli*, providing information on the population density of this bacterium while inhibiting the competition. Utilization of indole as an autoinducer might therefore be the reason for the lack of conservation of the AHL-dependent QS system in *E. coli*.

Short-chain fatty acids (SCFAs) and acetate/acetyl-CoA metabolism

In the caecum and in the large intestine, anaerobic bacteria ferment dietary fibers, producing a variety of metabolites, including short-chain fatty acids (SCFAs) such as propionate, butyrate, and acetate (den Besten et al., 2013). SCFAs are considered pivotal in the interactions between gut microbiota and the human host, as they seem to be a cofactor in important pathologies such as atherosclerosis, and neurological diseases such as autism (Galisteo et al., 2008; MacFabe, 2012). SCFAs absolute concentration and relative chemical composition vary with the diet and other factors (Macfarlane et al., 1992) creating an upward concentration gradient from the small towards the large intestine thus constituting a transit signal through the human gut (Herold et al., 2009).

Indeed, pathogenic EHEC regulate flagellar expression and motility based on relative composition and concentration of SFCAs by an unknown mechanism (Lackraj et al., 2016). Being an abundant and characteristic metabolite in the gut environment, SCFAs are also sensed by commensal *E. coli* through the histidine sensor kinase BarA, which in turn activates its cognate response regulator UvrY. The BarA/UvrY system controls the expression of the two non-coding RNAs CsrB and CsrC (Chavez et al., 2010), able to sequester the translational regulator CsrA, thus modulating carbon fluxes and inducing the expression of the PNAG polysaccharide. Moreover, expression of CsrB and CsrC is negatively regulated by cAMP-CRP (Pannuri et al., 2016). In addition to SCFAs, *E. coli* cells can also modulate biofilm formation in response to acetate; unlike other SCFAs, acetate is an endogenous product of *E. coli* fermentative metabolism, and is actively excreted from the cell to prevent its acidification. It has been proposed that, similar to SCFAs, extracellular acetate can induce the BarA/UvrY stimulating autophosphorylation of the BarA sensor protein. In addition, the UvrY regulatory protein can carry out autophosphorylation from acetyl phosphate, an intermediate of the mixed acid fermentation pathway in *E. coli* and other enterobacteria. Autophosphorylation of response regulators using acetyl phosphate might explain why *E. coli* strains mutated in the *ackA* genes, or *ackA pta* double mutants, both likely to accumulate acetyl phosphate, are more proficient in biofilm formation than wild type strain (Prüß et al., 2010). In addition to PNAG stimulation resulting from acetyl phosphate-dependent autophosphorylation of the UvrY regulator, this metabolite can trigger similar autophosphorylation activities in other response regulators, such as FimZ, RcsB and OmpR, independently of their cognate sensor proteins (Fredericks et al., 2006; Schwan et al., 2007; Wolfe et al., 2003), resulting in increased production of several biofilm determinants, namely, type 1 fimbriae and colanic acid (Fredericks et al., 2006; Wolfe et al., 2003). Thus, acetyl phosphate might represent a paramount example of how an intermediate of a metabolic pathway can relay a physiological cue to biofilm formation. It is noteworthy that acetyl phosphate can be produced in response to an excess of glucose or other sugars, as part of an overflow metabolism process, and could therefore serve as an indicator of the bacterial cell's energy state.

Polyamines

Polyamines, such as cadaverine, putrescine, spermine, spermidine, and norspermidine, are organic compounds that contain two or more amine groups and are found in abundance both in eukaryotic and prokaryotic cells. The major source of polyamines in the distal GI tract is the metabolism of the microbial flora, as polyamines are catabolites of basic amino acids such as arginine; polyamines synthesized by gut bacteria can be quickly absorbed by epithelial cells in the small intestine, providing yet another example of bacterial metabolites interacting with the host's cell (Milovic, 2001). In addition to *de novo* synthesis, bacteria have transporters for the uptake of extracellular polyamines. In *E. coli*, polyamines are essential for growth and viability, acting at various levels, from pH homeostasis, to stabilization of chromosomal DNA structure, to ribosomal activity: in particular, they appear to positively affect translation of several global regulators, including RpoS, Cya, H-NS, RelA and RpoZ (Terui et al., 2012). Thus, their physiological impact on the bacterial cell can be huge, and indeed they are connected with complex behaviors such as biofilm formation. While exogenous spermidine and norspermidine inhibit biofilm formed by potentially pathogenic *E. coli* in a concentration-dependent manner, putrescine greatly stimulates adhesion of nonpathogenic strains (Nesse et al., 2015; Sakamoto et al., 2012). Putrescine stimulation of biofilm is a concerted effect on different levels of the regulatory cascade. This polyamine promotes translation of UvrY and CpxR proteins, two response regulators belonging, respectively, to BarA/UvrY and CpxA/CpxR two-component response systems. Putrescine stimulates UvrY synthesis by promoting translation from the inefficient starting codon UUG of UvrY mRNA (Sakamoto et al., 2012). In turns, UvrY positively controls the expression of CsrB and CsrC small RNAs (Suzuki et al., 2002), which can sequester the CsrA protein, a translational repressor of the *pga* locus, necessary for poly-*N*-acetyl-glucosamine (PNAG) exopolysaccharide production, an important biofilm determinant (Wang et al., 2005; Wang et al., 2004). At the same time, putrescine induces a conformational change around the Shine-Dalgarno (SD) region of *cpxR* mRNA increasing its translational rate (Sakamoto et al., 2012). CpxR is a known transcriptional repressor of CsgD, thus inhibiting biosynthesis of curli adhesin (Prigent-Combaret et al., 2001). Although curli are mainly produced at conditions not

encountered in the human host (e.g., at low growth temperature) (Olsén et al., 1993), there is evidence that they are produced in the intestinal tract (Bokranz et al., 2005). Thus, rather than being a straightforward activator of biofilm formation, putrescine might promote a curli-PNAG switch as the main components of the biofilm matrix. So far, very little is known about the precise contribution of different biofilm determinants and the possible ecological meaning of their re-modulations of the biofilm matrix in the human GI tract. It is possible that coordinated production of different adhesion factors and biofilm determinants might be aimed at the colonization of different niches within the human host, and sensing of compounds such as polyamines might be one of the presiding mechanisms to this adaptation.

Intra/extracellular nucleotides and nucleobases (pyrimidines and uracil)

Several works have highlighted the importance of nucleotides and nucleobases, in particular uracil, for biofilm formation in both *E. coli* and *P. aeruginosa* (Garavaglia et al., 2012; Lee et al., 2008; Ueda et al., 2009). In *E. coli* K-12, inactivation of genes belonging to the *de novo* pyrimidine and purine biosynthesis totally impairs biofilm formation, while mutations leading to increased nucleotide biosynthesis result in biofilm stimulation (Garavaglia et al., 2012). The link between biofilm and intracellular nucleotide concentrations appears to be particularly strong, as even slight perturbations in the *de novo* nucleotide synthesis, albeit not affecting primary metabolism or cell growth, completely abolish biofilm formation and maintenance. The importance of intracellular nucleotide pools in biofilm formation is also suggested by the fact that antimetabolite drugs inhibiting *de novo* purine or pyrimidines biosynthesis, such as 5-fluorouracil, azathioprine, and sulfathiazole, are strong inhibitors of biofilm formation in *E. coli* and *P. aeruginosa* (Antoniani et al., 2010; Antoniani et al., 2013; Ueda et al., 2009). Azathioprine and sulfathiazole have been shown to inhibit c-di-GMP biosynthesis, suggesting that even slight perturbations in intracellular GTP concentrations, *i.e.*, the substrate of the diguanylate cyclase (DGC) enzymes responsible for c-di-GMP production, might impair their activity. Whether biofilm inhibition due to reduction in pyrimidine levels might also be mediated by c-di-GMP or possibly by other pyrimidine-derived nucleotides acting as signal molecules for biofilm formation,

remains to be addressed.

Free nucleotides, nucleosides, and nucleobases in the gut derive primarily from food degradation and up to 90% is absorbed by epithelial cells (Hess and Greenberg, 2012). However, fluctuations in their amounts can depend on bacterial metabolism: for example, uracil and hypoxanthine, both breakdown products of pyrimidine and purine nucleotide catabolism, accumulate in *E. coli* culture media during stationary phase (Rinas et al., 1995). Uracil was found to be actively excreted as a process to maintain pyrimidine homeostasis upon *de novo* synthesis dysregulation (Reaves et al., 2013), thus, accumulation of extracellular uracil might take place in response to changes in growth rate or carbon source availability, possibly acting as an indicator of metabolic activity. Moreover, it was recently shown that some gut pathogens secrete significant quantities of uracil while most commensal bacteria do not. In *Drosophila melanogaster* and other invertebrates used as model organisms for the study of innate immunity, uracil in the gut lumen strongly activates DUOX-dependent signaling, leading to reactive oxygen species release, as part of an immune response (Lee et al., 2013). Interestingly, exogenous uracil can specifically activate the YedQ diguanylate cyclase in *E. coli* K-12, resulting in stimulation of cellulose production (Garavaglia et al., 2012). These results suggest that pyrimidine sensing might indeed be tightly connected with c-di-GMP biosynthesis. In contrast, the YedQ homolog in the pathogenic bacterium *S. Typhimurium*, STM1987, was shown to modulate c-di-GMP synthesis in response to adenosine, L-arginine, L-lysine, *N*-acetyl-D-glucosamine, but not uracil (Mills et al., 2015). This specialization might reflect the different needs of a commensal versus an intracellular pathogen in the activation of c-di-GMP biosynthesis in response to specific cues.

N-acetyl-glucosamine

N-acetyl-glucosamine (GlcNAc) is an essential amino sugar necessary for peptidoglycan, lipopolysaccharide (LPS) and enterobacterial common antigen (ECA) biosynthesis in *E. coli*. GlcNAc is also the component of chitin, thought to be the biological polymer most abundant in nature, and is highly represented in glycosylated eukaryotic proteins and in glycoconjugates found on the surface of eukaryotic cells

(Varki and Varki, 2007). Due to its essentiality and its abundance, it is not surprising that most bacteria can obtain GlcNAc from the external environment through specialized uptake systems; however bacteria, including *E. coli*, can also synthesize GlcNAc using a *de novo* pathway. *E. coli* can also scavenge GlcNAc from recycling of its own extracellular peptidoglycan and LPS. Extracellular GlcNAc is turned into *N*-acetyl-glucosamine-6-phosphate (GlcNAc-6P) upon uptake and routed through an assimilatory pathway. Not surprisingly, such an important environmental signal is coupled to production of adhesion factors and biofilm formation: accumulation of GlcNAc-6P results in downregulation of curli *csg* operons by a mechanism independent of the NagC regulator (Barnhart et al., 2006), a repressor of the genes required for GlcNAc degradation. In contrast, GlcNAc-6P modulates NagC-dependent expression of FimB, a recombinase promoting the phase-variation switch necessary for the expression of genes encoding type I fimbriae (Pratt and Kolter, 1998). Accumulation of GlcNAc-6P turns off FimB activity, thus repressing type I fimbriae production (Sohanpal et al., 2004). Interestingly, FimB is controlled in a similar way by NanR and *N*-acetylneuraminate (Sohanpal et al., 2004), the predominant form of sialic acid present in humans. As *N*-acetylneuraminate is an environmental signal only found in the mammalian host, while GlcNAc/GlcNAc-6P levels affecting NagC activity can depend on the cell's energy state, *E. coli* can simultaneously regulate type I fimbriae and curli fibers production integrating both environmental and intracellular signals. Finally, the levels of both amino sugars increase in the host during inflammatory processes (Sillanauke et al., 1999), and can therefore act as indicators for the activation of an immune reaction. Consequently, *E. coli* can turn off curli and type I fimbriae, thus reducing antigenic load on the immune system (Sohanpal et al., 2004), in what appears to be a host-driven adaptive response in *E. coli* gene regulation.

Bile

Bile is a mixture of water, biliary acids, mostly composed of cholic acids derived from cholesterol salts, which are linked either to the amino acid glycine or to the organosulfonate taurine to form bile salts. Bile is a disinfectant agent and a potent detergent necessary for food digestion. It is secreted in the lumen of the duodenum from

the gall bladder upon food ingestion. Although Gram-negative bacteria like *E. coli* are intrinsically resistant to bile salts as they do not readily cross the outer membrane (Begley et al., 2005), they have evolved the ability to recognize bile as a signal in order to modulate adhesive structures production. Bile, in particular bile salts deoxycholate (DC) and cholate (CC), stimulate cellular adhesion in pathogenic *V. cholerae* through the modulation of vibrio polysaccharide (VPS) (Hung et al., 2006). Glucocholate hydrate (GCH) and DC induce expression of ETEC colonization factor CS5 (Nicklasson et al., 2012). In contrast, taurocholate (TC), *i.e.*, the bile salt constituted by the cholate and taurine complex, promotes *V. cholerae* biofilm dispersion by a passive mechanism based on extracellular matrix degradation and has no effect of colonization factor expression in CS5 expressing ETEC (Hay and Zhu, 2015; Nicklasson et al., 2012). In the pathogen *E. coli* O157:H7, bile salts activate the expression of virulence factors, iron acquisition genes and putative adhesive fimbriae, while regulating either positively or negatively different classes of flagellar genes (Hamner et al., 2013). In the closely related bacterium *Salmonella enterica* serovar Typhi, bile stimulates biofilm on gallstones through the production of a specific exopolysaccharide (EPS) encoded by the *yihU-yshA* and *yihV-yihW* loci, independently of the AfgD (CsgD) regulator (Crawford et al., 2008). The same genomic region is conserved in *E. coli* K-12, and it was recently described as involved in sulphoglycolysis (Denger et al., 2014); however, no direct link between biofilm formation and these genes in this or other commensal strains has been reported yet. In addition to gene regulation and possible induction of adaptive responses, bile has also been proposed to drive a genetic diversification in gut-inhabiting *E. coli* strains, leading to a non-motile subpopulation characterized by deletion mutations in the *flhDC* operon (De Paepe et al., 2011). It was suggested that selection would benefit bacteria growing in an environment characterized by high bacterial cell densities, such as biofilms.

One of the main component of human bile is taurine, an amino sulphonic acid that *E. coli* can utilize as a sulphur source if neither inorganic sulphate nor cysteine are present. Though inorganic sulphate is likely to be present in significant amounts in the intestinal tract, both cysteine and taurine are likely to be utilized as sulphur source by *E. coli*. Preferential utilization of sulphur sources regulates the inorganic sulphate

assimilation pathway and affects the intracellular levels of its intermediate phosphoadenosine 5'-phosphosulphate, which has recently been shown to affect production of curli and other adhesion factors (Rossi et al., 2014).

Temperature

Besides living in association with warm-blooded animals, Enterobacteria can be found in other natural niches, mostly in water environments. Thus, it is not surprising that temperature represents a key signal for sensing the transition between water environments, where temperature is usually low and variable, to the warm-blooded hosts, where it is higher and constant. In *E. coli*, the production of several adhesion factors is affected by temperature: indeed, curli fibers, cellulose, colanic acid and poly-*N*-acetylglucosamine (PNAG) are produced at higher levels at temperature below 30°C (environmental temperature) than at 37°C (host temperature) (Beloin et al., 2008; Gualdi et al., 2008; Olsén et al., 1993; Wang et al., 2004), thus suggesting that the biofilm mode might play a role in adaptation to conditions typically found outside the host. However, expression of other adhesins is not temperature-dependent or it is even enhanced at 37°C; it must be also considered that temperature regulation of adhesion factors can vary greatly, depending on strains and genetic backgrounds. Indeed, a number of works showed that, unlike laboratory strains, the matrix of different *E. coli* isolated from the gut at 37°C can be composed of a mix of curli fibers, cellulose (Bokranz et al., 2005; Saldaña et al., 2009), PNAG (Cerca and Jefferson, 2008; Pieper et al., 2013), while antigen 43 (Danese et al., 2000; Ulett et al., 2007) and type I fimbriae can mediate cellular adhesion at host temperature. In addition, most colonization factors of ETEC are induced at 37°C indicating that these virulence factors are needed for colonization of the host (Sjöling et al., 2007).

To date, biofilm control by temperature has mostly been studied for curli fibers and cellulose, in *E. coli* and *Salmonella* strains showing temperature dependence for their expression (Gerstel and Römling, 2003; Olsén et al., 1993). Although the precise mechanism governing curli temperature-dependent expression has not yet been fully elucidated, it might require converging regulatory circuits. One proposed mechanism involves the Crl protein, which promotes the association of the alternative sigma factor

RpoS to RNA polymerase; Crl expression is increased during the transition from exponential to stationary phase and it accumulates at low temperature (30°C), thus stimulating expression of *rpoS*-dependent genes, including the curli-encoding *csgBAC* operon (Bougdour et al., 2004). The second regulatory system depends on the small regulatory RNA DsrA. DsrA is stabilized at low temperatures, and in turn promotes RpoS translation under these conditions (Repoila and Gottesman, 2001). However, it was reported that the *csgBAC* operon can be transcribed in a *crl*- and *rpoS*-independent fashion (Arnqvist et al., 1994; Gualdi et al., 2007; Provence and Curtiss, 1992) and that DsrA might act on *rpoS* translation also at 37°C (Mandin and Gottesman, 2010), suggesting further regulatory mechanisms for temperature-dependent curli production. Since curli and cellulose synthesis are exquisitely dependent on c-di-GMP accumulation both in *E. coli* and in *Salmonella* (Römling, 2005), and expression of several DGC-encoding genes only takes place at low growth temperature (Sommerfeldt et al., 2009), this signal molecule might be the main responsible for temperature-dependent regulation of curli and cellulose.

However, curli are not modulated in the same way in response to temperature in other Enterobacteria. Indeed, especially pathogenic strains are able to express this kind of adhesins at higher temperature (37°C) during host invasion and colonization (Bian et al., 2000). Therefore, current data clearly outline the complexity of temperature regulation of curli fibers in *E. coli*, stressing that different strains might have evolved various adaptive strategies even within the same ecological niches.

Besides specifically controlling curli fibers and cellulose, temperature stimulates or inhibits biofilm formation in *E. coli* acting on the whole cell physiology through global regulators. Indeed, after temperature downshift, cold-shock proteins such as CspA, CspB, CspF, CspG and PNPase are strongly induced. CspA and its paralogues destabilize mRNA secondary structures, a process that may be crucial for efficient mRNA translation at low temperatures (Jiang et al., 1997). Even though specific targets of Csp proteins have not been completely identified, cold-shock proteins CspA, CspB, CspF, CspG and CspI can all work as positive regulators of biofilm formation (Domka et al., 2007). The RNA degradosome component PNPase was recently described as a negative regulator of poly-*N*-acetylglucosamine, thus suggesting control of this EPS in

response to temperature at RNA level (Carzaniga et al., 2012). Although the nine Csp proteins are homologues, their expression patterns greatly vary in different temperature range with CspA mostly expressed between 10-24°C, CspB and CspG at 15°C, and CspI between 10-15°C (Etchegaray et al., 1996; Wang et al., 1999), thus suggesting a different contribution in modulating biofilm formation during temperature shifts. On the contrary, two Cps proteins, CspE and CspC, are almost expressed at 37°C. The two proteins were shown to promote *rpoS* mRNA stabilization and expression at high temperature (Phadtare and Inouye, 2001), thus providing yet another mechanism coupling low growth temperature with expression of *rpoS*-dependent genes, which include in addition the curli-encoding *csg* operons and several diguanylate cyclases (Sommerfeldt et al., 2009).

Oxygen availability

Oxygen concentration in the intestine is generally considered to be low and bacteria face a microaerophilic condition (range 0.1 - 2.3%) (Levitt, 1971). In particular, the lumen of the small intestine and colon is typically anaerobic, whereas the brush border of the small intestine is considered microaerophilic, probably due to diffusion of oxygen from the capillary network at the tips of the villi (Sengupta et al., 2014). Moreover, while the biofilm develops and becomes thicker, the innermost cells experience anaerobic condition due to oxygen consumption of the more exposed parts of the biofilms, resulting in a rich and stratified environment with different cell phenotypes (Serra and Hengge, 2014).

Oxygen represents a major signal for *E. coli* biofilm development and anaerobic conditions negatively affect bacterial adhesion (Colón-González et al., 2004; Landini and Zehnder, 2002). While strict anaerobiosis is in general a negative signal for biofilm formation in *E. coli*, microaerophilic conditions can play a more complex role. Production of curli fibers is impaired in anoxic conditions through a direct oxygen sensing system, DosP/DosC. DosP is a c-di-GMP phosphodiesterase, while DosC is a diguanylate cyclase that synthesizes the second messenger c-di-GMP. Both proteins bind a heme prosthetic group and co-purify in a single macromolecular complex whose c-di-GMP net output depends on oxygen tension (Delgado-Nixon et al., 2000;

Tuckerman et al., 2011; Tuckerman et al., 2009). Tuckerman and colleagues suggested that decreasing O₂ concentration switches on DosC protein, while DosP activity is reduced resulting in a DosC-dependent c-di-GMP biosynthesis. However, totally anoxic conditions also impair DosC activity, suggesting that biofilm determinants might be strongly expressed in response to microaerophilic conditions only to be switched off in the absence of oxygen (Tagliabue et al., 2010b). In *E. coli*, the DosC/DosP protein complex was shown to control at least two different adhesion factors, namely curli fibers and PNAG (Tagliabue et al., 2010a; Tagliabue et al., 2010b), thus allowing coordinate production of two important biofilm determinants. Interestingly, although the DosC/DosP system is missing in the closely related bacterium *Salmonella*, curli fibers are produced at higher levels in response to microaerophilic conditions also in this bacterium (Gerstel and Römling, 2001), suggesting that a similar response might be mediated by different mechanisms in *E. coli* and *Salmonella*.

Cysteine/sulphate

As already mentioned, *E. coli* can assimilate sulphur from a broad range of sources such as inorganic sulfate, cysteine, aliphatic sulphonate (*i.e.* taurine), with cysteine as its preferred source (van der Ploeg et al., 2001). The gut lumen is a complex environment where different sources of organic sulfur are made available from processing of dietary proteins (*e.g.* cysteine and methionine) or from compounds deriving from the host (*e.g.* taurine, mucin). Taurine is very abundant in bile salts, as discussed in the section “*Bile*”, thus, sensing this sulphur source might be part of an adaptation to exposure to bile. In the absence of cysteine, its preferred sulphur source, *E. coli* scavenges inorganic sulfate, which is reduced to H₂S through the sulfate assimilation pathway, and subsequently used to synthesize L-cysteine (van der Ploeg et al., 2001). In addition to its role as a proteinogenic amino acid, cysteine is also used in other cellular processes including biosynthesis of methionine, coenzyme A, and glutathione, the main agent in maintenance of the redox state in the bacterial cell (Sekowska et al., 2000). Cysteine and sulfate metabolism are directly linked to biofilm formation in *E. coli*: inactivation of *cysB* gene, which is required for expression of all genes belonging to the sulphate assimilation pathway, stimulates biofilm formation of *E. coli* grown in rich media (Ren

et al., 2005) while a faint to null effect has been observed in casamino acids-based media (Ren et al., 2005; Rossi et al., 2014). Likewise, inactivation of *cysE* gene stimulates biofilm formation accelerating its development independently of flagella, colanic acid and type 1 fimbriae (Sturgill et al., 2004). The CysE enzyme catalyzes the biosynthesis of the metabolic intermediate *O*-acetyl-L-serine that, upon spontaneous conversion to *N*-acetyl-L-serine, acts as a positive inducer for CysB-dependent transcription regulation. Thus, loss of the CysE enzyme would affect CysB activity, resulting in the perturbation of sulphate reduction pathway intermediates, in particular, phosphoadenosine-5'-phosphosulphate (PAPS). Indeed, this molecule has recently been identified as a signal molecule connecting the sulphate/cysteine availability with biofilm formation by positively regulating production of curli and other adhesion factors (Rossi et al., 2014). Although the molecular mechanism is not clear, PAPS seems to act at two different levels, enhancing and/or synergizing the activity of the cAMP-CRP complex, at the same time providing a feedback mechanism through a putative interaction with the global regulator HU (Longo et al., 2016). In addition to controlling expression of curli fibers (Thomason et al., 2012; Zheng et al., 2004), cAMP-CRP complex directs the hierarchical utilization of different carbon sources (Kolb et al., 1993; Stülke and Hillen, 1998). Thus, PAPS could connect sulfate sensing with sugar metabolism (Longo et al., 2016). Indeed, additional links between sulphur sources, carbon utilization and biofilm formation is provided by the GlrKR two component system (also known as QseEF). In addition to responding to epinephrine/norepinephrine in EHEC strains, GlrK can also sense sulfate (Reading et al., 2009). In *E. coli* K-12, the cognate response regulator GlrR stimulates the expression of the small RNA GlmY, a positive effector for translation of GlmS (Göpel et al., 2014), a key enzyme in amino sugar biosynthesis (Badet et al., 1987). As discussed in a previous section, amino sugars, in particular *N*-acetylglucosamine, play a dual role in biofilm, as they are the building blocks of PNAG and other EPS acting as adhesion factors, and since intracellular *N*-acetyl-glucosamine-6-phosphate concentrations work as regulatory molecules for curli and fimbriae production (Barnhart et al., 2006; Itoh et al., 2008).

The reason why extracellular sulphate signaling should be routed through production of amino sugars and connected with carbon source sensing remains elusive. Unlike

eukaryotes, *E. coli* is unable to synthesize sulfated polysaccharides, which are however found in great abundance in the gut epithelia (e.g. mucin and sulphated glycosaminoglycans), where they carry out a protective function (Corfield et al., 2000). These carbohydrates can represent a valuable source of nutrients for bacteria, but usually their high sulfate content prevents their degradation; thus, expression of specific sulfatases is necessary to process host-derived sugars. Interestingly, YdeN and YdeM, two predicted sulfatases of *E. coli* K-12, are transcriptionally controlled by the level of *N*-acetyl-D-glucosamine-6-P (Oberto, 2010), another evidence for a connection between sulphate metabolism and amino sugars. Moreover, as mucin is composed of *N*-acetylgalactosamine, *N*-acetylglucosamine, fucose, galactose, and *N*-acetylneuraminic acid and traces of mannose and sulfate, it could be speculated that simultaneous presence of exogenous sulfate and *N*-acetylglucosamine (and/or *N*-acetylneuraminic acid) can be another indicator for *E. coli* to signal its location inside the host gut preparing the cell to utilization of host-derived sugars.

5 The host's defense against *E. coli* biofilm formation

Curli fimbriae are a virulence factor in several disease situations. Using synthetic molecules interfering with curli biogenesis (curlicides), curli were shown to promote the pathogenesis of urinary tract infection in a mouse model (Cegelski et al., 2009). Interestingly, the endogenous antimicrobial peptide LL-37 exhibits a similar function. Indeed, we could show that LL-37 interferes specifically with the formation of biofilms at subinhibitory concentrations (Kai-Larsen et al., 2010). By binding to CsgA monomers, polymerization and curli formation is inhibited *in vitro* and biofilm formation *ex vivo* is strongly reduced. In addition, curliated strains are protected from the antimicrobial activity of LL-37. As LL-37 binds to isolated curli, presumably, the peptide is trapped in the extracellular net of fibers and thus non-functional. However, LL-37 might also act through additional mechanisms in the cytoplasm. Engineered peptides related to LL-37 specifically inhibit biofilm formation by interference with ppGpp second messenger signaling in various bacterial species including *E. coli* (de la Fuente-Nunez et al., 2014).

Other consequences, potentially, include interference with the quorum sensing systems in *P. aeruginosa* (Overhage et al., 2008).

Besides interacting with LL-37, curli induce a strong host immune response and promoted early colonization, but also bacterial elimination by neutrophils at a later stage; in contrast, the biofilm extracellular matrix component cellulose opposed these effects (Kai-Larsen et al., 2010). Further, curli can trigger immune activation via Toll-like receptors (Tükel et al., 2010) and curli-DNA composites, at least from *S. Typhimurium*, stimulate autoimmunity in mice (Gallo et al., 2015).

Currently, it is now known whether LL-37 interferes with the functionality of additional fimbriae. As initiation of infection and immunostimulation are a general hallmark of curli fimbriae, interference with biofilm formation by antimicrobial peptides such as LL-37 might be an anti-virulence strategy also on other mucosal surfaces (Chromek et al., 2012).

6 Second messenger signaling regulating biofilm formation

The nucleotide second messengers cyclic AMP, cyclic di-GMP and ppGpp have been shown to regulate biofilm formation in *E. coli*. The environmental stimuli of these nucleotide-based second messengers are fundamentally different, as cyclic AMP is regulated in response to sugar availability, while cyclic di-GMP regulates sessile/motile and commensal/virulence life style transition through a multitude of extra- and intracellular signals, whereas adaptation of ppGpp concentration occurs through nutrient starvation. Nevertheless, though, these signaling systems are interconnected at several knots (Fig. 5).

Catabolite repression of *rdar* and other biofilm types is partially mediated through the cAMP/CRP system by different sugars at high concentration, while low concentration of the same molecules has a biofilm-stimulating effect (Jackson et al., 2002; Prüß et al., 2006; Sutrina et al., 2015). Although some additional receptors have been identified, the cAMP signaling system is essentially linear with one cAMP synthase, the cAMP receptor protein, a transcriptional regulator, which regulates over 100 genes, and one cAMP phosphodiesterase.

In contrast, the cyclic di-GMP signaling system consists of over 30 membrane standing or cytoplasmic c-di-GMP metabolizing proteins plus a number of strain/genomic island/pathovar specific enzymes (Povolotsky and Hengge, 2015; Römling, 2005). The main biofilm activator CsgD is a major target of transcriptional regulation by cyclic di-GMP signaling and cyclic di-GMP subsequently activates production of downstream extracellular matrix components curli fimbriae and cellulose (Povolotsky and Hengge, 2012). The fundamental sessility/motility life style transition of cyclic di-GMP as in other bacteria exists also in *E. coli*, thus inversely regulated with biofilm formation is motility (Römling, 2012; Römling et al., 2013). Besides in rdar biofilm formation, ETEC strains show elevated cyclic di-GMP in interaction with intestinal epithelial cells (Kansal et al., 2013). In the AIEC strain LF82, cyclic di-GMP inhibits type 1 pili synthesis as well as adhesion, and invasion through expression of the FliA dependent phosphodiesterase YhjH (Claret et al., 2007). Interestingly, type 1 fimbriae are repressed by cyclic di-GMP, as indirectly concluded by the effect of the metabolizing enzymes, in this strain, while, conventionally, cyclic di-GMP elevates production of type 1 fimbriae in the UTI strain background as would be expected (Spurbeck et al., 2012). Also synthesis of the commonly produced exopolysaccharide PNAG, involved in attachment (Agladze et al., 2005), depends on c-di-GMP levels (Steiner et al., 2013). The diguanylate cyclase YdeH (DgcZ) regulates PNAG production (Boehm et al., 2009). Transcription of *ydeH* depends on the two component system CpxAR (Raivio et al., 2013) activated by the lipoprotein NlpE upon surface recognition (Otto and Silhavy, 2002). Thereby, YdeH connects surface sensing with subsequent adhesion (Lacanna et al., 2016).

ppGpp, long called an alarmone, is actually a second messenger responding to nutrient deprivation thereby activating the ‘so-called’ stringent response. One pathway of ppGpp mediated activation of biofilm formation, is enhancing expression of the small RNAs CsrB and CsrC, thereby inhibiting CsrA (Fig. 5; (Dalebroux and Swanson, 2012)). CsrA subsequently regulates the expression of cyclic di-GMP metabolizing proteins, thereby connecting ppGpp and cyclic di-GMP signalling (Jonas et al., 2008). In a *csrA* deletion background, though, ppGpp inhibits biofilm formation (Boehm et al., 2009), indicating counteracting regulatory mechanisms by ppGpp.

7 Perspectives and future questions

In summary, *Escherichia coli* commensal strains and the various *E. coli* pathovars frequently form biofilms. Thereby biofilm formation is highly variable. On the other hand, the probiotic strain Nissle 1917 shows a highly stable biofilm pattern. Thus, what are the evolutionary forces in commensalism and disease, but also outside the human body, which shape biofilm regulation? And, more specifically, what is the physiological impact of the different *rdar* biofilm regulatory patterns seen e.g. among commensal *E. coli* strains?

Indeed, we have discovered few biofilm phenotypes; can we define additional phenotypes and the circumstances, when they are required? The commensal model organism *Escherichia coli* K-12 has seven additional chaperone usher pathway fimbriae in addition to type 1 fimbriae (Korea et al., 2010), which seem to be functional and mediate biofilm formation. Under which biofilm conditions are these fimbriae expressed? For example, does *E. coli* interact with other microorganisms of the gut microflora through biofilm formation?

Also, how are the different biofilm phenotypes, as they exist, differentially regulated? Adhesion and biofilm factors specific to *E. coli* pathovars exist. How many biofilms are regulated by the ubiquitous second messenger cyclic di-GMP network? And if regulated, how is differential regulation achieved? Horizontally acquired fimbriae such as Pap and Saf fimbriae specific for certain *E. coli* pathovars, adjacently include regulon specific cyclic di-GMP turnover proteins (Sjöström et al., 2009). Besides cyclic di-GMP signalling, also other secondary messenger signalling systems are involved in biofilm regulation such as the ppGpp and cAMP signalling system. Also, phosphotransfer signalling through two component systems and the phosphoenolpyruvate phosphotransferase systems (PTS) that differentially phosphorylate proteins in response to carbohydrate transport over the cytoplasmic membrane are involved in biofilm formation. Are these systems relevant, and if so, how are these systems interconnected in *E. coli*?

Although not covered extensively in this review, we have to state that cyclic di-GMP signaling is a major regulator of biofilm-motility and prospectively biofilm-virulence transition in *E. coli* (Spurbeck et al., 2012), as it is in other bacteria (Römling, 2012). Can

we define specific cyclic di-GMP metabolizing enzymes involved in biofilm versus virulence regulation in the different pathovars? How many of the described signals affecting biofilm formation interact with the cyclic di-GMP signalling pathway, either directly, by being the ligand of sensor domains, or indirectly?

In general, few phenotypes mediated by cyclic di-GMP signaling have been identified in *E. coli*; how can we identify additional phenotypes regulated by cyclic di-GMP signaling? Acute virulence phenotypes are generally inhibited in the biofilm life style and by cyclic di-GMP signaling, is this also the case in *E. coli*? In addition, we know very little about the contribution of biofilm formation and cyclic di-GMP signaling to chronic infection phenotypes.

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Figure legends

Fig. 1 Typical biofilm models as formed by commensal *E. coli* strains. The commensal strains were previously isolated from the faeces of healthy humans (Bokranz *et al.*, 2005). A: Macrocolony biofilm appearance on agar plates. Upper panel: Rdar (red, dry and rough) biofilm morphotype of strain Fec51 (left) and saw (smooth and white) non-biofilm forming Fec75 (right) grown at 28°C for 72 h on plates supplemented with 40 µg/ml Congo Red and 20 µg/ml Coomassie Brilliant Blue G. Fec51 rdar biofilm formation is due to the production of an extracellular matrix consisting of curli fimbriae and cellulose (Römling, 2005). In contrast, Fec75 does not express these components and expresses a saw morphotype. Expression of cellulose can also be visualized on Calcofluor supplemented (50 µg/ml) LB agar plates without salt (lower panel). Fec51 fluoresces after excitation with 365 nm UV light. CR: Congo Red; CF: Calcofluor (Fluorescent brightener 28).

B: Pellicle formation at the air-liquid interface in standing culture. Top: pellicle of commensal strain Fec101, down: commensal strain Fec75 (negative control). Bacterial strains were grown in a 96 well plate in LB without salt medium at 28°C for 48h.

C: Biofilm formation in liquid culture as exemplified by clumping and adherence to abiotic glass surface. Fec101 was grown in LB without salt medium at 28°C for 18h with shaking at 200rpm. Under these growth conditions, extensive clumping and adherence to wall of well is observed (left). To stain the attached cells, the liquid was removed and the biofilm stained with 0.4% crystal violet for 10 min (right).

D: Biofilm formation of commensal *E. coli* TOB1 on HT-29 epithelial cells; left, adherence of a biofilm producing mutant; right, adherence of a non-biofilm producing mutant ((Wang *et al.*, 2006), picture: Heinrich Lünsdorf).

Fig 2 Biofilm formation of *E. coli* in the gastrointestinal tract. A. *E. coli* forms biofilms in the environment potentially following the common biofilm developmental pathway as depicted. Attachment and biofilm formation to produce causes food-borne outbreaks. A motile cell (A) is attaching reversibly (B) to a surface. After becoming irreversibly attached (C) a microcolony is formed, expressing an extracellular matrix (D). After full maturation of the biofilm (E), dispersion takes places in that motile cells (F) or microcolonies (G) leave the biofilm. B. Biofilm formation of *E. coli* on the intestinal epithelium. Commensal *E. coli* are mainly present in the mucus layer of the colon establishing biofilms by formation of an extracellular matrix consisting of curli and cellulose. Enterohemorrhagic *E. coli* (EHEC) attach to the epithelial cells via the use *E. coli* common pilus ECP, hemorrhagic pilus HCP, as well as mucin binding flagella and other adhesins. Intimate attachment is facilitated by interaction of intimin with the translocated Tir receptor or intimin-host nucleolin interaction, followed by pedestal formation and attaching and effacing (A/E) lesions. Enteroinvasive *E. coli* (EIEC) pass both mucus layers and invade the epithelial cells. Enteroaggregative *E. coli* EAEC form a biofilm using aggregative adherence Dr-fimbriae (AAF) as well as dispersin. Typical enteropathogenic *E. coli* tEPEC form A/E lesions as well as pedestals similar to EHEC. The EspA filament promotes initial attachment, the adhesin LifA facilitates attachment along with bundle-forming pili (BFP), supported by *E. coli* common pilus (ECP) and mucin binding flagella. Intimate attachment is mediated by Tir-intimin interaction.

Enterotoxigenic *E. coli* (ETEC) adhere to the epithelial lining via the adhesin EtpA exposed at the flagellar tip and colonization factors (CFs) of different kinds. Further, the secreted protein CexE might have a similar function as dispersin in EAEC. Intimate attachment occurs by protein based adhesion via Tia and TibA. Diffusely adherent *E. coli* DAEC adhere non-localized to the epithelial cells through (Dr-) fimbrial and afimbrial (Afa) adhesins. Adherent-invasive *E. coli* (AIEC) attach by type I pili supported by flagella. Further, adhesion is mediated by interaction of the bacterial chitinase ChiA with the host chitinase 3-like-1 (CHI3L1) via chitin binding domains. Long polar fimbriae promote invasion.

Fig 3 The phylogroups of *E. coli* and their correlation to pathotypes. The phylogroups A and B1 are sister groups with a common origin. Pathogens that induce watery diarrhea such as ETEC, EPEC and EAEC often belong to these phylogroups. The apparently most successful lineages of UPEC and EHEC O157/H7 belong to phylogroups B2 and E, respectively. There are numerous exceptions and individual ETEC, EPEC EAEC, and UPEC strains, as well as successful lineages such as ETEC O169 and EHEC O111 and O26 may belong to different phylogroups. However, there might be advantages assigned with certain chromosomal backgrounds that fit better with certain virulence factors than others and thus shape virulence including biofilm formation, transmission pathways and infection capabilities. The lines are not to scale and do not represent the genetic differences between phylogroups.

Fig 4 The regulatory network involved in adhesive structure production in the gastrointestinal (GI) tract. Extracellular and intracellular signals that are involved in biofilm formation of commensal *E. coli* strains in the GI tract. The figure only represents a selection of the many cues that might affect biofilm formation. For clarity, some regulatory mechanisms, such as those depending on small RNAs, have been omitted. A. Bacterial-derived signals. *E. coli* senses and communicates using auto-released signals, known as quorum sensing (QS) molecules: AI-1 can be perceived but not synthesized, while AI-2 and AI-3 are also synthesized by specific pathways. B. Environmental and metabolic signals deriving from metabolism of either *E. coli* or gut microbiota or eukaryotic cells. Solid lines: experimentally verified; dashed lines: unknown mechanism if connecting two regulators, or intermediate reactions omitted if connecting metabolites. green lines: positive effectors, red lines: negative effectors, orange lines: post-transcriptional stimulatory effect. Cellulose and PNAG production are represented by dotted lines as not all *E. coli* isolates synthesize them.

Fig 5 Interconnection of second messenger signalling cAMP, c-di-GMP and ppGpp signalling to regulate biofilm formation in *E. coli*. For more detailed description, see text. TA=Toxin/Antitoxin system.

Figure 1

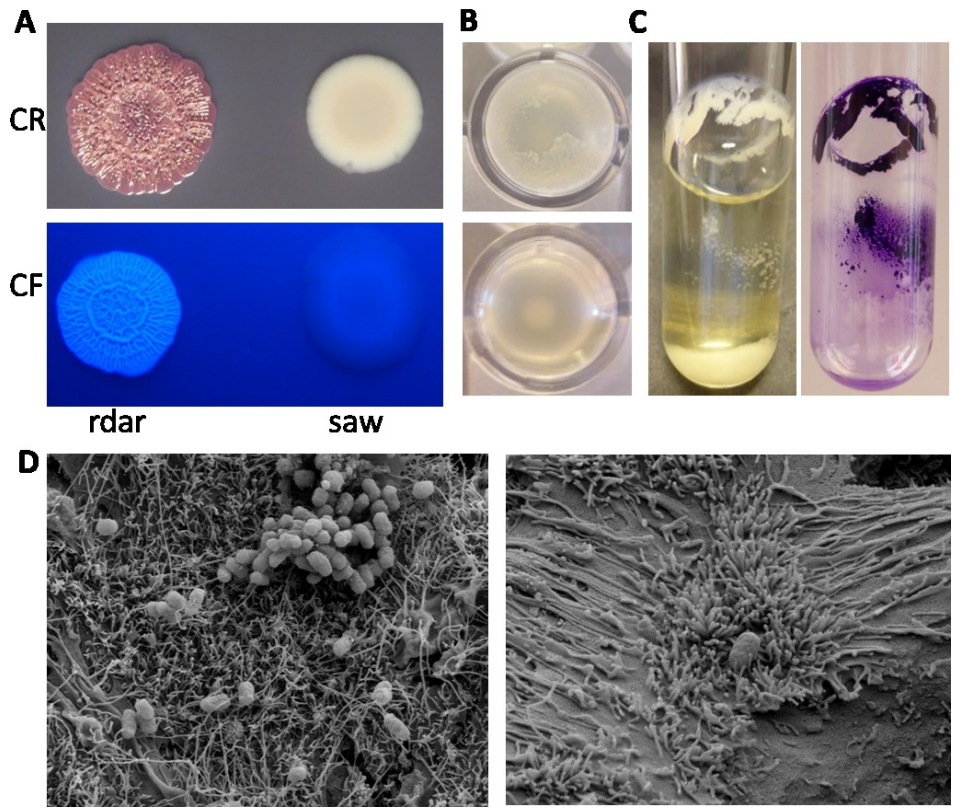


Figure 2

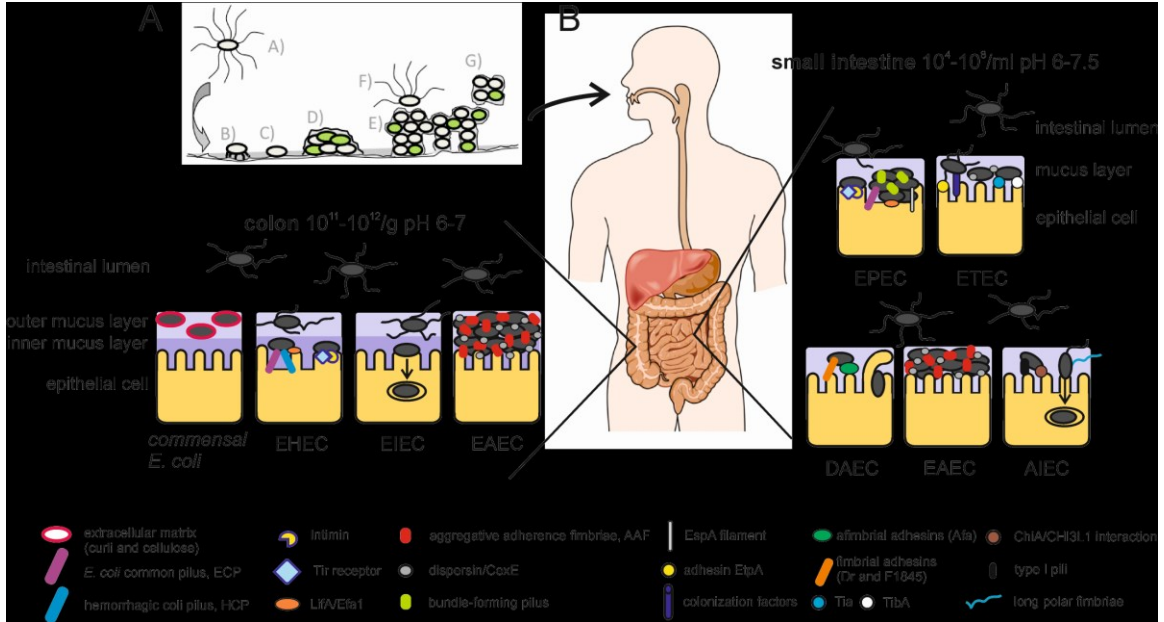


Figure 3

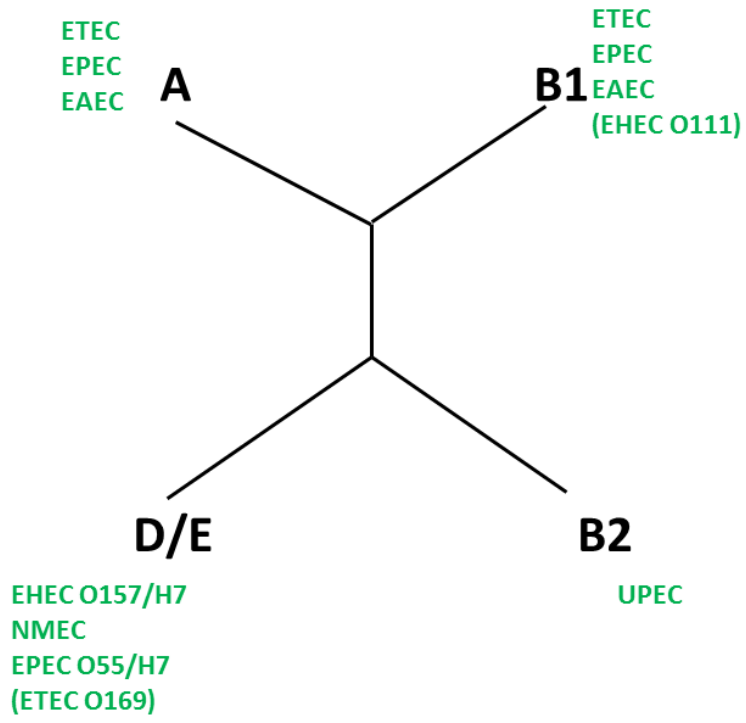
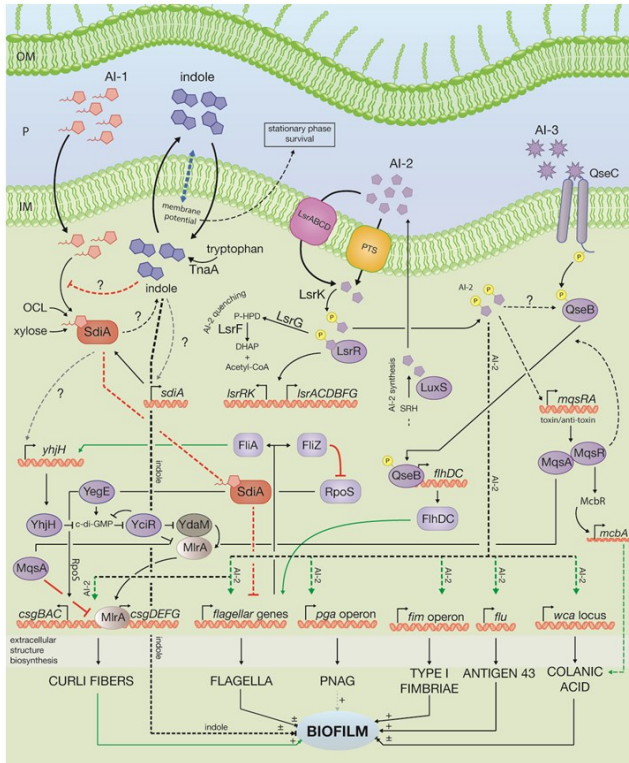


Figure 4

A



B

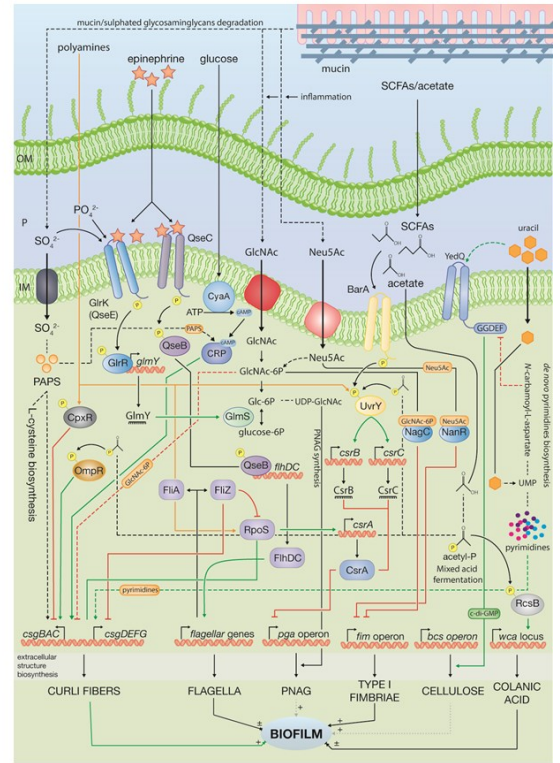


Figure 5

