Molecular Determinants of Surface Colonisation in Diarrhoeagenic Escherichia coli (DEC): from Bacterial Adhesion to Biofilm Formation

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Abstract

Escherichia coli is primarily known as a commensal colonising the gastrointestinal tract of infants very early in life but some strains being responsible for diarrhoea, which can be especially severe in young children. Intestinal pathogenic *E. coli* include six pathotypes of diarrhoeagenic *E. coli* (DEC), namely the (i) enterotoxigenic *E. coli*, (ii) enteroaggregative *E. coli*, (iii) enteropathogenic *E. coli*, (iv) enterohemorragic *E. coli*, (v) enteroinvasive *E. coli*, and (vi) diffusely-adherent *E. coli*. Prior to human infection, DEC can be found in natural environments, animal reservoirs, food processing environments and contaminated food matrices. From an ecophysiological point of view, DEC thus deal with very different biotopes and biocoenoses all along the food chain. In this context, this review focuses on the wide range of surface molecular determinants acting as surface colonisation factors (SCFs) in DEC. In the first instance, SCFs can be broadly discriminated into (i) extracellular polysaccharides, (ii) extracellular DNA, and (iii) surface proteins. Surface proteins constitute the most diverse group of SCFs broadly discriminated into (i) monomeric SCFs, such as autotransporter (AT) adhesins, inverted ATs, heat-resistant agglutinins or some moonlighting proteins, (ii) oligomeric SCFs, namely the trimeric ATs, and (iii) supramolecular SCFs, including flagella and numerous pili, *e.g.* the injectisome, type 4 pili, curli chaperone-usher pili or conjugative pili. This review also details the gene regulatory network of these numerous SCFs at the various stages as it occurs from pre-transcriptional to post-translocational levels, which remains to be fully elucidated in many cases.

One-sentence summary

Diarrhoeagenic *Escherichia coli* (DEC) express numerous surface colonisation factors contributing to their contamination of the food chain, from natural environments, animal reservoirs, food processing environments to food matrices, and ultimately, human infection.

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Introduction

Most recent phylogenetic analyses have revealed that the *Escherichia* genus is subdivided into eight groups containing three species, namely *Escherichia coli*, *E. fergusonii*, and *E. albertii*, as well as five clades numbered from I to V (Lawrence & Hartl, 1991, Walk *et al.*, 2009). *E. coli* is undoubtedly the most investigated bacterial species and is used as a model organism in microbiology. This lipopolysaccharidic (LPS) diderm bacterium (archetypical Gram-negative bacterium) is primarily known as a harmless commensal of the gastrointestinal tract (GIT) (Mason & Richardson, 1981, Chagnot *et al.*, 2013). While *E. coli* is prevalently an inhabitant of the gut of warm-blooded animals, especially mammals but also birds, it is worth mentioning this bacterial species can also be isolated from fish, frogs or reptiles, such as crocodiles, turtles or snakes, but also insects, such as flies (Janisiewicz *et al.*, 1999, Souza *et al.*, 1999, Gordon & Cowling, 2003, Escobar-Paramo *et al.*, 2006, Blazar *et al.*, 2011); *E. coli* generally appears more prevalent in herbivores and omnivores than carnivores. In humans, *E. coli* colonises the GIT of young children early in life and usually represents less than 1 % of the human intestinal microbiota in adults (Eckburg *et al.*, 2005).

Nevertheless, some *E. coli* species possess some virulence factors that enable them to cause a broad range of human extraintestinal and intestinal infections. On one side extraintestinal pathogenic *E. coli* (ExPEC) mainly comprises the uropathogenic *E. coli* (UPEC), neonatal meningitis *E. coli* (NMEC), necrotoxic *E. coli* (NTEC) and sepsis-associated *E. coli* (SEPEC). On the other side, and in addition to the adherent invasive *E. coli* (AIEC) associated with Crohn's disease (Mann & Saeed, 2012), the intestinal pathogenic *E. coli* (InPEC) essentially encompasses six pathotypes of diarrhoeagenic *E. coli* (DEC), namely the (i) enterotoxigenic *E. coli* (ETEC), (ii)

enteroaggregative *E. coli* (EAEC), (iii) enteropathogenic *E. coli* (EPEC), (iv) enterohemorragic *E. coli* (EHEC), (v) enteroinvasive *E. coli* (EIEC), and (vi) diffusely-adherent *E. coli* (DAEC) (Kaper *et al.*, 2004, Croxen & Finlay, 2010); of note, EHEC belong to the larger group of shigatoxin-encoding *E. coli* (STEC), or shigatoxin-producing *E. coli*, which are not all considered as pathogenic as they can exhibit very various virulence levels ranging from avirulence to hyper-virulence (Karmali *et al.*, 2003, Laing *et al.*, 2009, Monteiro *et al.*, 2016). The pathogenicity of DEC strains is well documented and their main virulence factors are also well defined (Croxen & Finlay, 2010). Some of these pathotypes are not restricted to human infections, but can be responsible for diarrhoea in animals, for instance (i) ETEC in porcines (piglets), bovines (calves) or ovines (lambs), (ii) EPEC in rabbits, dogs, cats, pigs, calves, lambs and goats, and (iii) STEC in calves and piglets (Beutin, 1999, DebRoy & Maddox, 2001); to date, EAEC, EIEC and DAEC have not been reported as etiological agents of diarrhoea in animals. Despite the high genome plasticity demonstrating intensive gene flow, the population structure of *E. coli* remains mostly clonal (Touchon *et al.*, 2009), with a clear delineation into seven principal phylogenetic groups (A, B1, B2, C, D, E and F) (Jaureguy *et al.*, 2008, Walk *et al.*, 2009, Tenaillon *et al.*, 2010, Clermont *et al.*, 2013, Beghain *et al.*, 2018). Commensal *E. coli* strains generally belong to phylogroup A, whereas DEC usually belong to phylogroups A, B1, C, D and E (Jaureguy *et al.*, 2008, Okeke *et al.*, 2010, Croxen *et al.*, 2013, Hazen *et al.*, 2016, Rossi *et al.*, 2018): (i) ETEC can be found in phylogroups A and B1 and to lesser extent in D, (ii) EAEC are found within phylogroup A but also B1, D and to a smaller extend in B2, (iii) EPEC can belong to phylogroups E and B2, (iv) EHEC strains are mostly found in phylogroups B1 and D but also in E (with the with serotype O157:H7 or O104:H4), (v) EIEC are mainly present in phylogroups A, B1 and E, together with *Shigella*, which are

138 essentially *E. coli* species from phylogenetic and taxonomic perspectives (Brenner *et*
139 *al.*, 1972, Lan & Reeves, 2002, Chaudhuri & Henderson, 2012, Pettengill *et al.*, 2015),
140 and (vi) DAEC which mostly belong to phylogroups B2 and D (Servin, 2014, Mosquito
141 *et al.*, 2015, Walczuk *et al.*, 2019). This distinct grouping suggests a parallel evolution
142 of the different pathotypes on multiple occasions, possibly with the intervention of
143 mobile elements enabling the acquisition of specific combinations of virulence factors
144 (Chaudhuri & Henderson, 2012, Croxen *et al.*, 2013).

145 DEC can be found all along the food chain (Giaouris *et al.*, 2014, Kim *et al.*,
146 2017). They can have various environmental reservoirs, such as ruminants for EHEC,
147 and are mainly transmitted to humans by the faecal-oral route through the consumption
148 of contaminated food, including water, or contact with contaminated surfaces (Croxen
149 *et al.*, 2013). Besides anthropozoonosis, transmission can also occur from host to host
150 between humans. In any case, the colonisation of the food chain by DEC is a major
151 issue for the agri-food and public health sectors alike. The surface colonisation process
152 can occur *via* bacterial adhesion and/or biofilm formation to various biotic or abiotic
153 surfaces. When the reversible adhesion to the surface by low energy linkages (e.g.
154 electrostatics, Van der Waals interactions) is overcome, some bacteria can grow at the
155 surface. As such, biofilm formation can be broadly defined as the sessile development
156 of microorganisms at a surface or interface (Azeredo *et al.*, 2017). Biofilm can be
157 monospecies but are more generally multispecies in the natural environment, forming
158 a complex multicellular community, which is often embedded in an exopolymeric
159 matrix (EPM) (Costerton, 1995, Costerton *et al.*, 1999). It confers to bacterial cells an
160 increased resistance against environmental stress, antibiotics and/or immunological
161 defences of the host. Once the reversible adhesion is overcome, the bacterial biofilm
162 formation is *per se* divided in several steps: (i) initial and irreversible adhesion of

bacterial cells to the surface, (ii) bacterial division at the site of adhesion resulting in the formation of microcolonies, (iii) maturation of the biofilm architecture into a three-dimensional structure, and (iv) bacterial dispersion enabling the colonisation of other sites (O'Toole *et al.*, 2000, Hall-Stoodley & Stoodley, 2002). Biofilm formation can thus plays a key role in DEC ecophysiology by enabling colonisation of various environmental niches (soil, water, vegetables, agri-food surfaces, *etc*…), the asymptomatic and direct colonisation of some hosts, as well as contributing to transmission through the food chain and ultimately human infection (Ahmed *et al.*, 2013).

Most information about the colonisation process in *E. coli* is focused on the domesticated laboratory strain K12, commonly considered as representative of the *E. coli* species (Beloin *et al.*, 2008). However, this notion is biased due to the numerous and very significant genotypic and/or phenotypic differences with commensal and pathogenic *E. coli* isolates (Hobman *et al.*, 2007). Indeed, *E. coli* K12 has one of the smallest genomes compared to other genome-sequenced strains of *E. coli* due to the loss of a large variety of genes during its domestication (Lenski, 2017). With regards to the selective pressures that shapes the genome evolution, *E. coli* K12 have been replicated and studied for a long time under laboratory conditions, far from those encountered in natural environments (Hobman *et al.*, 2007); some molecular determinants, including some surface colonisation factors (SCFs), could thus be lacking or misregulated in domesticated laboratory strains of *E. coli* compared to commensal and pathogenic *E. coli* isolates. As the interface between the bacterial cell and its surroundings, the molecular surface determinants are key players in the initial adhesion and sessile development processes and this review aims at summarising exhaustively the SCFs present in DEC. The complexity of the regulation network occurring at

various stages, from pre-transcriptional to post-translocational levels, is also highlighted. A greater understanding of the parameters that influence adhesion and biofilm formation may inform the development of interventions to minimise DEC dissemination in the food chain, from the environment, animal, food, to human.

1. Molecular determinants involved in surface colonisation by DEC

The colonisation processes along the food chain, from natural environments, such as soil, plants and animals, to food environments, including the industrial processing food chain and food matrices, and ultimately infection or asymptomatic carriage in human, are very complex and involves many molecular determinants. Sessile development at a surface or interface is generally accompanied by the formation of an EPM embedding the bacterial cells in biofilms (Figure 1). These exopolymers can act as glue for adherence of the bacterial cell to the support and shape the architecture of the biofilm (Hobley *et al.*, 2015). Furthermore, the EPM provides protection by shielding the bacteria from desiccation and antimicrobial compounds but also participates in the channelling of nutrients and signalling molecules (Sutherland, 2001, Starkey *et al.*, 2004, Beloin *et al.*, 2008). As such, the EPM contribute to the survival strategy and persistence of bacteria in various environmental conditions (Branda *et al.*, 2005). Molecular determinants participating in the surface colonisation by DEC can either be closely associated with the bacterial cell surface and form the cell-associated EPM (caEPM) or present in the extracellular milieu, namely the interstitial EPM (iEPM) (Figure 1) (van Houdt & Michiels, 2005).

At a biochemical level, EPM components can be broadly discriminated between (i) extracellular polysaccharides (EPS), (ii) extracellular DNA (eDNA), and (iii) surface proteins. Depending on the different DEC pathotypes, these various determinants can be either present or absent (Table 1). Outer membrane vesicles (OMVs) have been reported to be components of the EPM in *E. coli* K12 (Schooling & Beveridge, 2006) and their presence in biofilm from DEC is likely, although it remains to be demonstrated. To date, there is no report of their contribution to biofilm formation in DEC, as observed in *Pseudomonas aeruginosa* or *Helicobacter pylori* (Yonezawa *et al.*, 2009, Wang *et al.*, 2015), but it is an aspect that would deserve further investigation in DEC. Of note, poly-γ-glutamate (PGA) can be found as a component of the EPM of numerous bacteria, especially parietal monoderm bacteria (archetypical Gram-positive bacteria) and only a few LPS-diderm bacteria, where it can either be released or cell-surface attached to form a capsule (Candela & Fouet, 2006, Ogunleye *et al.*, 2015, Radchenkova *et al.*, 2018) but, to date, this has never been reported in any *E. coli* strain.

1.1. Exopolysaccharides (EPS)

EPS are one of the main components of the EPM in *E. coli* biofilms (Beloin *et al.*, 2008). DEC can biosynthesise a variety of EPS, namely (i) lipopolysaccharide (LPS), (ii) poly-β-1,6-N-acetyl-D-glucosamine (PNAG), (iii) colanic acid, and (iv) cellulose. Because of their intimate association with the bacterial cell surface, several of these EPS can contribute to the caEPM and the formation of a so-called capsule. Actually, *E. coli* harbours some serotype-specific polysaccharides, namely lipopolysaccharides (LPS) (O antigen) and capsular polysaccharides (K antigen). *E. coli* capsules are composed of high-molecular weight polysaccharides embedding the bacterial cells and linked to the cell-surface *via* covalent attachments (Whitfield,

2006). More than 80 capsular antigens have been reported in *E. coli*, which are divided into four groups, from G1 to G4 (Whitfield, 2006, Yaron & Romling, 2014). DEC (including EPEC, ETEC and EHEC) produce G1 and G4 capsules that share a common assembly system and can be associated with the lipid A of LPS (K_{LPS}) or be structurally similar to the O-polysaccharides of the LPS (O-antigen capsules). During an infection, these capsules allow bacteria to be protected from opsonophagocytosis and complement-mediated killing (Whitfield, 2006). In EHEC O104:H4, the capsule has been shown to play a role in bacterial survival in the environment and in direct bacterial interaction with plants (Jang & Matthews, 2018).

1.1.1. Lipopolysaccharide (LPS)

LPS is located at the outer leaflet of the outer membrane (OM) and part of the caEPM (Raetz & Whitfield, 2002). This glycolipidic polymer is formed around a toxic component, lipid A, and for this reason is also considered an endotoxin; the LPS is further composed of the core region linked to the lipid A (divided into an inner and outer part) and the O-antigen that is linked to the outer part of the core region (Raetz & Whitfield, 2002). Biosynthesis and assembly pathways of LPS have been fully described and involve more than 50 genes encoded in operons or monocistrons scattered on the bacterial chromosome (Sandkvist, 2001, Szalo *et al.*, 2006). The structures of lipid A and its core region are highly conserved in *E. coli* but the core region has five basic structures, called R1, R2, R3, R4 and K12. Among these, R1 is the most prevalent in non-STEC clinical isolates of *E. coli* and R3 is more associated with STEC strains (Gibb *et al.*, 1992, Appelmelk *et al.*, 1994, Currie & Poxton, 1999, Amor *et al.*, 2000). In *E. coli* clinical isolates, R1 is most prevalent, whilst the K12 core is not detected (Gibb *et al.*, 1992, Appelmelk *et al.*, 1994). More than 170 O-antigens

have been identified and consist of 10–25 repeating units containing one to eight sugar residues (Stenutz *et al.*, 2006). The O-antigen can be present (smooth LPS, also called S-LPS or LPS I, resulting in colonies with a smooth phenotype) or absent (rough LPS, also called R-LPS or LPS II, resulting in colonies with a rough phenotype) depending on the *E. coli* strain; if the core region is also absent, it is called deep-rough LPS (Hitchcock *et al.*, 1986). Smooth strains are the most commonly found in nature, including in DEC, whereas the rough phenotype is more commonly found in laboratory strains (Whitfield & Keenleyside, 1995, Nataro & Kaper, 1998). For smooth strains, the LPS length is positively correlated with the force of adhesion (Strauss *et al.*, 2009). The O-antigen assists adhesion through hydrogen binding (Tomme *et al.*, 1996). For example, it has been demonstrated that the O-antigen enables EHEC O157:H7 strains to colonise animal hosts (Sheng *et al.*, 2008). Mutations in LPS biosynthesis genes have been shown to affect the adhesion of *E. coli* to abiotic surfaces and its biofilm formation ability (Bilge *et al.*, 1996, Genevaux *et al.*, 1999, Landini & Zehnder, 2002, Beloin *et al.*, 2006). Additionally, LPS can promote or inhibit biofilm formation by two distinct mechanisms, mainly by interacting with cell-surface-exposed adhesion factors. It has been shown that alteration of LPS synthesis can impair type 1 pili and colanic acid expression as well as bacterial motility, whereas the reduction in LPS expression may unmask *E. coli* adhesins and thus promote adhesion or biofilm formation as observed for EHEC O157:H7 strain (Bilge *et al.*, 1996, Beloin *et al.*, 2006, Beloin *et al.*, 2008).

1.1.2. Poly-N-acetyl glucosamine (PNAG)

PNAG is an EPS attached to the bacterial surface and is involved in biofilm formation on abiotic surfaces (Wang *et al.*, 2004). The biosynthetic pathway for PNAG is encoded by the *pgaABCD* locus (formerly *ycdSRQP*). Initiation of PNAG production

occurs with the PgaDC, a glycosyl transferase localised on the cytoplasmic side of the inner membrane that uses the UDP-N-acetyl-D-glucosamine as substrate (Wang *et al.*, 2004, Itoh *et al.*, 2005, 2008). The PNAG polymer is exported and anchored to the bacterial surface through the *β*-barrel formed by two outer membrane proteins (OMPs), namely PgaB and PgaA. Although PNAG forms a surface capsule and is one of the main components of the caEPM in diverse bacterial biofilm, the *pga* locus is not present in all *E. coli* strains (Cerca *et al.*, 2007, Cimdins *et al.*, 2017). In DEC, PNAG plays a role in the stabilisation of biofilm architecture (Wang *et al.*, 2004, Al Safadi *et al.*, 2012). It has been demonstrated to be important for biofilm formation of EHEC on sprouts and tomato roots (Matthysse *et al.*, 2008). *In vivo* expression of *pgaA* during infection by EHEC O104:H4 suggests that biofilm formation is a key step in pathogenesis (Al Safadi *et al.*, 2012). PNAG is also expressed by some ETEC strains and often induced by conditions found in the environment (Gonzales-Siles & Sjoling, 2016).

1.1.3. Colanic acid

Colanic acid is a negatively charged polymer of glucose, galactose, fucose, and glucuronic acid produced by most *E. coli* strains, including DEC (Obadia *et al.*, 2007). The *wca* operon (or *cps*) encodes 19 proteins including polymerases involved in colanic acid synthesis from sugar residues (Stevenson *et al.*, 1996). Colanic acid actually forms the G1 capsule but a significant portion of the colanic acid produced can also be released into the extracellular milieu to contribute to the iEPM (Whitfield & Roberts, 1999, Beloin *et al.*, 2008, Beloin *et al.*, 2008). The exact contribution of colanic acid to biofilm formation is still unclear (Matthysse *et al.*, 2008, May & Okabe, 2008). Nonetheless, it forms a physical barrier that helps bacteria to survive outside the host

with the formation of a protective capsule around the bacterial cell. This capsule allows *E. coli* biofilms to resist osmotic and oxidative stresses as well as to temperature variations (Whitfield & Roberts, 1999, Chen *et al.*, 2004). In EHEC O157:H7, it has been shown to play a role in the bacterial survival in simulated GIT fluids (Mao *et al.*, 2006). In EAEC, the presence of colanic acid has been linked with the formation of large biofilm structures on the surface of sprouts (Borgersen *et al.*, 2018). In contrast, the production of colanic acid could also mask some cell-surface adhesins and consequently impair initial adhesion to some supports (Hanna *et al.*, 2003, Schembri *et al.*, 2004, Beloin *et al.*, 2008).

1.1.4. Cellulose

Cellulose is a linear homopolysaccharide composed of D-glucopyranose units linked by β-1→4 glycosidic bonds. While this widespread biopolymer is generally related to plant biology, it is also present in the iEPM in some bacterial species where it plays a role in protection, maturation and structure of the biofilm (Solano *et al.*, 2002, Ude *et al.*, 2006). In *E. coli*, cellulose biosynthesis genes are located in two operons, namely *bcsQABZC* and *bcsEFG* (Zogaj *et al.*, 2001, Solano *et al.*, 2002, Le Quere & Ghigo, 2009). The cellulose synthase is formed by BcsAB, which catalyses cellulose biosynthesis from UDP-glucose subunits and forms a transmembrane pore across the inner membrane for cellulose export prior to secretion across the OM *via* a β-barrel pore formed by BcsC (Keiski *et al.*, 2010, Omadjela *et al.*, 2013). The role of the *bcsEFG* operon is still unclear but its presence is necessary for cellulose production (Solano *et al.*, 2002). These genes are found in both commensal and pathogenic *E. coli* strains (Beloin *et al.*, 2008). Although cellulose production is essential for biofilm maturation, over-production negatively impacts biofilm formation and bacterial

aggregation, possibly by coating and thus masking the adhesive properties of surface proteins such as curli (Gualdi *et al.*, 2008). In EHEC O157:H7 and EPEC O127:H6 cellulose production has been shown to contribute to biofilm formation, and consequently, host colonisation and survival in different environments (Saldana *et al.*, 2009). The involvement of cellulose in *E. coli* colonisation of plant materials has also been demonstrated but it depends on the vegetable, as its presence seems dispensable for biofilm formation by *E. coli* O157:H7 to spinach leaves, but it is required for bacterial adhesion to alfalfa sprouts (Matthysse *et al.*, 2008, Macarisin *et al.*, 2012). Expression of these genes in some ETEC strains is often induced at ambient temperatures, low ionic strength and nutrient limitation (Bokranz *et al.*, 2005, Szabo *et al.*, 2005).

1.2. Extracellular DNA (eDNA)

The importance of eDNA in biofilm maturation has been demonstrated in numerous bacterial species (Muto & Goto, 1986, Kadurugamuwa & Beveridge, 1995, Steinberger *et al.*, 2002), including *E. coli* (Xi & Wu, 2010, Nakao *et al.*, 2012). As a component of the iEPM, eDNA serves as structural component of the biofilm but can also contribute to a cation gradient, as a nutrient source, induce antibiotic resistance, and aid horizontal gene transfer (Bockelmann *et al.*, 2006, Palchevskiy & Finkel, 2006, Sanchez-Torres *et al.*, 2011). However, the role of eDNA in DEC strains remains to be elucidated. The molecular mechanism explaining the presence of eDNA has been a subject of investigation for some time but essentially results from the release of genomic DNA upon cell lysis, following the bacteriophage lytic cycle or bacterial cell apoptosis (Palmen & Hellingwerf, 1995, Steinmoen *et al.*, 2002, Qin *et al.*, 2007). Nonetheless, the lysis of outer membrane vesicles (OMVs) containing DNA

(Kadurugamuwa & Beveridge, 1996, Whitchurch *et al.*, 2002), as well as DNA secretion through the conjugative Type IV, subtype b, secretion system (T4bSS) (Hamilton *et al.*, 2005, Chagnot *et al.*, 2013) could also contribute to the presence of eDNA. The extent and respective contribution of these different mechanisms to the presence of eDNA would undoubtedly require further investigations, especially in DEC, also considering the impact of the apparent presence of pancreatic nuclease in the intestine (Maturin & Curtiss, 1977).

1.3. Cell-surface proteins

The cell surface of LPS-diderm bacteria can display a number of proteins associated with the OM. Proteinaceous determinants found at the bacterial cell surface and acting as SCFs can be broadly discriminated into (i) monomeric proteins, (ii) multimeric proteins (Figure 2).

In the scientific literature, *E. coli* adhesins have generally been discriminated between fimbrial and afimbrial (or non-fimbrial). However, and as with animal classification, a group is much better defined by features that are present rather than by the absence of some features. As such, the term afimbrial adhesins does not tell anything about the nature of these adhesins. In addition, some afimbrial adhesins later appeared to be atypical fimbriae secreted by the same family of protein secretion system, e.g. the CS31A (coli surface associated 31a antigen) pili (Adams *et al.*, 1997). For these reasons, we here propose to regroup those cell-surface proteins under the term of monomeric proteinaceous adhesins, or monomeric proteinaceous colonisation factors. Besides, the term fimbriae is not very well defined across the Bacteria kingdom when considering different bacterial species. On the contrary, the term pili can be used as a generic term encompassing the various type of pili and fimbriae, including curli or

injectisome. In addition, some cell-surface appendages contributing to surface colonisation in bacteria cannot be categorised as fimbrial adhesins *per se*, e.g. the flagella and the trimeric autotransporters. To avoid any ambiguity, these different cell-surface appendages are proposed to be regrouped under the term of multimeric proteinaceous colonisation factors.

1.3.1. Monomeric proteinaceous surface colonisation factors

In *E. coli*, monomeric protein acting as SCFs include some autotransporters (ATs), inverted autotransporters (IATs), and some OMPs, but also the surface-exposed lipoprotein SslE, Efa-1 (*E. coli* factor adherence 1), dispersin, as well as some moonlighting proteins. Of note, the ATs (also sometimes called classical ATs) only belong to the Type V, subtype a, secretion system (T5aSS) and correspond to monomeric polypeptides with modular organisation into at least three main regions, i.e. (i) a N-terminal signal peptide, (ii) a central passenger, and (iii) a translocator at the C-terminus (Desvaux *et al.*, 2003, Desvaux *et al.*, 2004, Leo *et al.*, 2012). ATs (T5aSS) should not be mistaken with the trimeric ATs, hybrid ATs and inverted ATs, which belong the T5sSS, T5dSS and T5eSS, respectively.

1.3.1.1. Autotransporters (ATs)

Classical ATs acting as SCFs comprise the autotransporter adhesins (ATAs), the self-associating autotransporters (SAATs), and some serine protease autotransporters from Enterobacteriaceae (SPATES) (Henderson & Desvaux, 2004, Henderson *et al.*, 2004, Desvaux *et al.*, 2006, Rojas-Lopez *et al.*, 2017).

1.3.1.1.1. Autotransporter adhesins (ATAs)

ATAs enable direct adhesion to abiotic supports, e.g. glass, stainless steel or plastic ware, and/or biotic surface, e.g. mammalian cells or extracellular matrix (ECM) components such as collagens (Vo *et al.*, 2017). As such, they can also belong to MSCRAMM (microbial surface components recognizing adhesive matrix molecules) proteins (Chagnot *et al.*, 2012).

In EHEC, several enterohaemorrhagic *E. coli* autotransporters (Eha) have been identified (Wells *et al.*, 2008). Among them, EhaB has been shown to promote bacterial cells binding to laminin and collagen I (Wells *et al.*, 2008, Wells *et al.*, 2009), whereas EhaJ causes strong adherence to fibronectin, fibrinogen, collagens II, III and V, and laminin (Easton *et al.*, 2011). EhaB has also been identified in EPEC and ETEC (Zude *et al.*, 2014). Immediately adjacent to the *eha* gene, *egtA* encodes a glycosyltransferase. EhaJ requires glycosylation to mediate strong biofilm formation but not for adhesion to ECM components (Easton *et al.*, 2011). Following genomic analysis, *ehaJ* appears to be also present in EAEC, EIEC and ETEC where its function is still unknown. In EPEC, its exact function in the colonisation process remains unclear, as it does not seem to be required for bacterial adhesion and biofilm formation (Easton *et al.*, 2011). While EhaD has been shown to mediate biofilm formation, its role in bacterial adhesion has not been determined yet and its contribution to sessile development in DEC would require more in-depth investigation (Wells *et al.*, 2008). In the laboratory strain *E. coli* K12, the EhaD homologue YpjA has been shown to promote adhesion to glass and polyvinyl chloride (PVC), as well as biofilm formation together with the EhaC homologue YfaL and YcgV (Roux *et al.*, 2005). In EHEC, however, EhaC was not shown to promote biofilm formation (Wells *et al.*, 2008). A homologue of *ycgV* has been genetically identified in several DEC, namely EPEC, ETEC, EAEC and EIEC (Wells *et al.*, 2010,

Zude *et al.*, 2014). Altogether, this information emphasises the need for further experimental characterisation of the adhesive functions of Eha, particularly considering the diversity of DEC.

Some ATs originally identified in UPEC and acting as adhesins have been identified in DEC, namely UpaB (uropathogenic *E. coli* autotransporter B) and UpaI (Zude *et al.*, 2014). From UPEC investigations, these proteins appeared to promote adhesion to a wide range of ECM components (Allsopp *et al.*, 2012, Zude *et al.*, 2014), whilst UpaI was further demonstrated to mediate biofilm formation (Zude *et al.*, 2014). Although the genes are found in EPEC and STEC, none of them have been functionally characterised in any DEC to date (Zude *et al.*, 2014).

Following genomic analysis, AatA (avian pathogenic *E. coli* autotransporter A) appears to be also present in some DEC strains (Zude *et al.*, 2014). In APEC (avian pathogenic *E. coli*), AatA is important for pathogenesis as it enhanced adhesion to chicken fibroblast cells (Dai *et al.*, 2010, Li *et al.*, 2010, Wang *et al.*, 2011). However, its role and contribution in DEC is still unknown.

1.3.1.1.2. Self-associating autotransporters (SAATs)

SAATs are primarily enable to associate to one another resulting in bacterial cell autoaggregation (Klemm, 2006). In *E. coli*, the SAATs regroup ATs from the Ag43 (antigen 43), AIDA-I (adhesin involved in diffuse adherence phenotype) and TibA (toxigenic invasion locus b) families (Trunk *et al.*, 2018). Of note, SAATs differentiate from ATAs as they do not necessarily play a role in direct adhesion to biotic or abiotic surfaces but can nonetheless contribute directly or indirectly to surface colonisation.

Ag43 is probably the SAAT which has triggered the most research to date, with most of the information resulting from investigations in the *E. coli* K12 laboratory

strain (van der Woude & Henderson, 2008). Besides autoaggregation, Ag43 has been demonstrated to increase biofilm formation on abiotic surfaces (Kjaergaard *et al.*, 2000) and adhesion to epithelial cells (Sherlock *et al.*, 2006, de Luna *et al.*, 2008) but to decrease bacterial motility (Ulett *et al.*, 2007). The gene encoding Ag43 has been shown to be highly expressed during the early stage of biofilm formation (Schembri *et al.*, 2003) but not in mature biofilms (Beloin *et al.*, 2004). While biofilm formation is favoured by the autoaggregation phenomenon (van der Woude & Henderson, 2008), Ag43 is not involved in gut colonisation (de Luna *et al.*, 2008). It is also known that the expression of pili would shield the interaction between Ag43 and thus prevent the autoaggregation (Korea *et al.*, 2010). Phylogenetic analysis revealed the *agn43* gene is distributed into two subfamilies, namely subfamily I (SF-I) and SF-II, and is only found among, but not all, *E. coli* (including some *Shigella* spp.) (van der Woude & Henderson, 2008). It has been suggested that *agn43* is more prevalent in pathogenic *E. coli* strains than in commensal *E. coli* strains (van der Woude & Henderson, 2008). It can be detected as a single gene copy, like in *E. coli* K12, or in multiple alleles, like in EHEC O157:H7 EDL933 where two identical copies are found in two different pathogenicity islands, namely the O-island 43 (OI-43) and OI-48 (Torres *et al.*, 2002). In UPEC CFT073, Ag43 is encoded by two different alleles, namely *agn43a* and *agn43b* (Ulett *et al.*, 2007). Compared to the Ag43 encoded by the first allele, Ag43 from allele b had a slower autoaggregation kinetics and lower propension for biofilm formation.

Autoaggregation results from the L-shape structure of Ag43 passenger region, which drives molecular interaction *via* salt bridges and hydrogen bonds along the β-helix structure in a molecular Velcro-like handshake mechanism (Heras *et al.*, 2014). In *E. coli* O157:H7 EDL933, Ag43 was shown to promote autoaggregation, calcium

473 binding and biofilm formation but was unable to mediate adhesion to epithelial cells
474 (Torres *et al.*, 2002). While present in other DEC, such as EPEC, ETEC and EAEC
475 (Zude *et al.*, 2014, Vo *et al.*, 2017), functional characterisation of Ag43 in these
476 different pathotypes has not be examined in details to date. Most recently, phylogenetic
477 network analysis revealed the Ag43 passengers were distributed into four distinct
478 classes, namely C1, C2, C3 and C4 (Ageorges *et al.*, 2019). Structural alignment and
479 modelling analyses indicated the N-terminal and C-terminal regions of the passengers
480 belonged to two different subtypes which gave rise to these four distinct Ag43 classes
481 upon domain shuffling. Functional analyses demonstrated that expression of Ag43 ^{C3}
482 (which both *agn43a* and *agn43b* from UPEC CFT073 belong to) induced a slower
483 sedimentation kinetics of bacterial cells and smaller aggregates compared to the three
484 other Ag43 classes (Ageorges *et al.*, 2019). Using prototypical Ag43 ^{C1} from *E. coli*
485 K12 MG1655, Ag43 ^{C2} from EHEC EDL933, Ag43 ^{C3} from UPEC CFT073 (allele
486 *agn43b*) and Ag43 ^{C4} from ETEC H10407, it appeared that heterotypic interactions
487 occurred in a very limited number of cases compared to homotypic interactions. This
488 ability of Ag43 variants to specifically identify genetic copies of themselves in other
489 bacterial cells through Ag43-Ag43 interactions further suggests a greenbeard effect
490 (Gardner & West, 2010, Wall, 2016), the ecophysiological relevance of which
491 undoubtedly require further investigation (Ageorges *et al.*, 2019).

492 AIDA-I is involved in the diffuse adherence of DEC strains (Benz & Schmidt,
493 1989, Benz & Schmidt, 1992) and also in bacterial autoaggregation, biofilm formation
494 and adherence to a wide range of human and non-human cells (Benz & Schmidt, 1989,
495 Sherlock *et al.*, 2006). While the function of AIDA-I is quite similar to Ag43, they
496 clearly belong to different protein families (Vo *et al.*, 2017). The gene encoding AIDA-
497 I is especially prevalent in ETEC and STEC strains from porcine origin, which suggests

498 pork as a main animal reservoir for this gene (Niewerth *et al.*, 2001, Ha *et al.*, 2003).
499 In EPEC, the AIDA-I gene (*aidA*) is associated with *aah* which encodes a 45-KDa
500 heptosyltransferase (Benz & Schmidt, 2001). These genes are plasmid located and
501 transcribed as bicistronic mRNA, but their expression seems to be restricted to a small
502 number of DEC strains (Owen *et al.*, 1996, Sherlock *et al.*, 2004). Aah (adhesin
503 associated heptosyltransferase) modifies the AIDA-I by addition of 19 heptose residues
504 on average, which enables EPEC to adhere to human cells (Benz & Schmidt, 1992,
505 Benz & Schmidt, 2001, Laarmann & Schmidt, 2003, Schembri *et al.*, 2004). In EHEC
506 O157:H7, though, AIDA-I does not play a role in adherence to cultured cells or to pig
507 intestinal epithelial cells (Yin *et al.*, 2009). This suggests different subfamilies or
508 classes of AIDA-I could exist as observed for Ag43, which would require further in-
509 depth investigation.

510 TibA has been found to self-aggregate, promote biofilm formation and facilitate
511 colonisation of the intestinal epithelia (Sherlock *et al.*, 2005, Cote & Mourez, 2011). In
512 ETEC, TibA is encoded by the *tib* operon, which also encodes the glycosyltransferase
513 TibC (Lindenthal & Elsinghorst, 1999). Glycosylation of TibA is important for its
514 function since its unglycosylated form is less stable and cannot oligomerise properly
515 and in turn cannot promote bacterial adhesion to epithelial cells (Cote *et al.*, 2013);
516 nonetheless, it can autoaggregate, promote biofilm formation and cell invasion.
517 Interestingly, TibA, AIDA-I and Ag43 have been reported to interact with one another
518 resulting in the formation of mixed bacterial aggregates (Klemm, 2006). These
519 interesting findings deserve further in-depth characterisation, especially with regards to
520 recent findings where the interactions between Ag43 variants appears quite specific
521 (Ageorges *et al.*, 2019).

In *E. coli* O157:H7, EhaA has been shown to mediate autoaggregation and adhesion to primary epithelial cells derived from the bovine terminal rectum, as well as biofilm formation (Wells *et al.*, 2008). As such, EhaA can be considered as an additional member of SAAT also found in EAEC, EPEC and ETEC (Vo *et al.*, 2017). Similarly, UpaC was reported to promote autoaggregation, as well as biofilm formation (Zude *et al.*, 2014). UpaC is found in a wide range of InPEC (Zude *et al.*, 2014). Of note, some ATAs such as UpaI can further promote autoaggregation to some extent (Zude *et al.*, 2014).

1.3.1.1.3. Serine protease autotransporters from enterobacteriaceae (SPATEs)

SPATEs correspond to a subfamily of protease autotransporters that specifically exhibit a serine protease domain (IPR034061) in the passenger region (Rojas-Lopez *et al.*, 2017). While their primary function is associated with the degradation of various proteins, such as mucin or haemoglobin, they can contribute to bacterial virulence *via* their cytotoxic effect, and some can even be involved in bacterial colonisation (Dautin, 2010).

In EHEC, EspP (extracellular serine protease plasmid-encoded), also known as PssA (protein secreted by Stx-producing *E. coli*), contributes to biofilm formation, bacterial adherence to intestinal epithelial cells, including bovine primary rectal cells, and colonisation of the bovine intestine (Dziva *et al.*, 2007, Puttamreddy *et al.*, 2010, Farfan & Torres, 2012). EspP is encoded on the pO157 plasmid and can be found in diverse STEC isolates (van Diemen *et al.*, 2005, Dziva *et al.*, 2007, Ruiz-Perez & Nataro, 2014). At the bacterial cell surface, EspP passenger domains self-assemble to form supramolecular structures, called ropes (Xicohtencatl-Cortes *et al.*, 2010). Besides cytopathic activities, the EspP ropes have strong adhesive properties to host epithelial

cells and can further serve as a substratum for bacterial adherence and biofilm formation. Similar observations have also been made for EspC from EPEC (Xicohtencatl-Cortes *et al.*, 2010).

In EAEC, Pic (protein involved in colonisation) is involved in mucin degradation but also directly in mucin binding (Gutierrez-Jimenez *et al.*, 2008, Andrade *et al.*, 2017). It thus participates in intestinal colonisation and may also be involved in bacterium-mucus biofilm (Navarro-Garcia & Elias, 2011). Pic is also expressed by the hybrid EHEC/EAEC *E. coli* O104:H4 but its exact contribution to the colonisation process in this genetic background remains to be ascertained (Henderson *et al.*, 1999, Harrington *et al.*, 2009, Abreu *et al.*, 2015, Abreu *et al.*, 2016). Of note, Shmu is a mucinase identical to Pic found in *Shigella* (Rajakumar *et al.*, 1997).

1.3.1.2. Inverted autotransporters (IATs)

In IATs, which correspond to the Type V, subtype e, secretion system (T5eSS), the translocator is located in the N-terminal region and the passenger at the C-terminal, which is the opposite of the modular organisation found in ATs (Tsai *et al.*, 2010, Oberhettinger *et al.*, 2012). In DEC, there are several IATs acting as SCFs, namely intimin, FdeC (Factor adherence of *E. coli*) and YeeJ. More recently, additional IATs have been identified in *E. coli*, where *iatA* appeared quite prevalent but the functional characterisation of the gene product is still awaited (Goh *et al.*, 2019). IatB, IatC and IatD from an environmental *E. coli* strain were further shown to be involved in strong biofilm formation when overexpressed in a recombinant *E. coli* K12 background, but not in autoaggregation nor adhesion to ECM proteins (Goh *et al.*, 2019). While identified in several DEC, their role and contribution in their native genetic background is still unknown.

1.3.1.2.1. Intimin

Intimin is the prototypical member of IATs (Leo *et al.*, 2015). In EPEC and EHEC, the intimin is encoded by the *eae* (for *E. coli* attachment effacement) gene in the locus of enterocyte effacement (LEE) (Nataro & Kaper, 1998). This protein interacts specifically with its receptor Tir (translocated intimin receptor) allowing the establishment of the intimate attachment of the bacteria with the host cell, pedestal formation and attaching/effacing lesions (A/E) (Schmidt, 2010). In addition, intimin contributes to intestinal colonisation in a Tir-independent manner (Mallick *et al.*, 2012). Intimin may also bind to alternative receptors such as β_1 integrins or nucleolin but this remains to be clarified (Liu *et al.*, 1999, Leo *et al.*, 2015).

1.3.1.2.2. Factor adherence of *E. coli* (FdeC)

FdeC is a widespread IAT in *E. coli* and present in all DEC pathotypes (Nesta *et al.*, 2012, Easton *et al.*, 2014). In EHEC O26:H11, FdeC was shown to contribute to biofilm formation and potentially in colonisation of the terminal rectum of cattle (Easton *et al.*, 2014).

1.3.1.2.3. YeeJ

More recently, the gene encoding YeeJ has been reported to be present in some DEC, namely EHEC, EPEC, ETEC and EIEC (Martinez-Gil *et al.*, 2017). In *E. coli* K12, this IAT has been shown to participate in biofilm formation. While YeeJ exists into two distinct variants of different lengths, no functional difference could be detected between them. However, the contribution of YeeJ to biofilm formation in DEC remains to be established.

1.3.1.3. Other outer membrane proteins (OMPs)

Besides ATs and IATs, several additional monomeric OMPs can act as SCFs in DEC, namely OmpA, Hra (Heat-resistant agglutinin), and Iha (Iron-regulated protein A homologue adhesin). OMPs are integrated to the OM *via* the β-barrel assembly machinery (Bam) complex (Leyton *et al.*, 2015, Botos *et al.*, 2017, Schiffrin *et al.*, 2017).

1.3.1.3.1. Outer membrane protein A (OmpA)

While originally considered as a pore forming protein (Sugawara & Nikaido, 1992), whether the OmpA β-barrel offers a channel for the continuous passage of water or solutes remains controversial (Smith *et al.*, 2007). Nowadays, OmpA is rather viewed as a multifaceted protein with functions of an adhesin as well as an invasin. In EHEC O157:H7, OmpA is involved in adhesion to intestinal epithelial cells (Torres & Kaper, 2003, Kudva *et al.*, 2015). OmpA further appears to be the key molecular determinant for bacterial adhesion to plant surfaces, such as alfalfa sprouts (Torres *et al.*, 2005). The role of OmpA as an invasin was demonstrated in NMEC (Prasadarao *et al.*, 1996) but remains to be established in DEC. Interestingly, OmpA can be encoded by at least two different alleles, namely *ompA1* and *ompA2* (Power *et al.*, 2006). Many of the interaction properties of OmpA emanate from protein loops external to the OM, which are displayed on the bacterial cell surface (Smith *et al.*, 2007); in the two alleles, differences in these regions could influence the adhesin and/or invasin properties of the protein. Of note, OmpA further serves as a receptor for bacteriophages and bacteriocins (Smajs *et al.*, 1997, Power *et al.*, 2006). Regarding biofilm formation, the direct contribution of OmpA remains controversial; while OmpA from *E. coli* K12 has been shown to bind to abiotic surfaces and to significantly influence biofilm formation (Lower *et al.*, 2005, Barrios *et al.*, 2006), the role of OmpA in EHEC O157:H7 biofilm

formation appears to be minor and it acts rather as a modulator than a contributor to sessile development (Torres *et al.*, 2005, Kudva *et al.*, 2015). Keeping in mind that OmpA is an important contributor to the structural integrity of the bacterial cell envelope by bridging the OM and cell wall, along with lipoproteins (Wang, 2002), the interpretations of phenotypes from OmpA mutants must be considered with caution due to possible pleiotropic effects that can be confounding. Further investigations on these various aspects are clearly needed, and in particular the allelic variation of OmpA should also be more carefully considered to decipher their exact role.

1.3.1.3.2. Heat-resistant agglutinin (Hra)

The Hra family of OMPs were first described with Hek (haemagglutinin from *E. coli* K1) in NMEC, where it was reported to promote autoaggregation, interactions with human erythrocytes and epithelial cells, as well as adhesion to, and invasion of epithelial cells (Fagan & Smith, 2007). Hek was originally identified because of its homology with Tia (toxigenic invasion protein A) (Bhargava *et al.*, 2009). In ETEC, Tia mediates attachment to intestinal epithelial cells as well as their invasion (Fleckenstein *et al.*, 1996, Sjoling *et al.*, 2015). It also appears to bind several mammalian heparan sulphate binding proteins suggesting, that ETEC use these ubiquitous cell surface heparan sulphate proteoglycans as receptors to adhere and invade host epithelial cells (Fleckenstein *et al.*, 2002).

In EAEC O42, Hra1 (heat-resistant agglutinin 1) was demonstrated to be responsible for autoaggregation and aggregative adherence, as well as biofilm formation (Bhargava *et al.*, 2009). While these observations were made upon protein expression in nonadherent and nonpathogenic laboratory *E. coli* strains, an EAEC 042 *hra1* deletion mutant was not deficient in these phenotypes, indicating that Hra1 is an accessory colonisation factor in this genetic background. While *hra1/hek* was originally

643 considered absent from DEC but restricted to UPEC, NMEC and sepsis *E. coli*
644 (Dobrindt *et al.*, 2002, Cooke *et al.*, 2010), it later became clear that *hra1* and *tia* are
645 common among DEC, especially EAEC but also EPEC (Fleckenstein *et al.*, 1996,
646 Mancini *et al.*, 2011). In the EAEC strain 60A, Hra2 it is not involved in
647 autoaggregation or invasion, but only in adherence to epithelial cells (Mancini *et al.*,
648 2011); its involvement in bacterial adhesion to abiotic supports and biofilm formation
649 remains to be elucidated. The prevalence of *hra2*, however, seems to be very low
650 among DEC.

651 More recently, a novel member of the Hra family has been identified in STEC,
652 namely Hes (Hemagglutinin from shigatoxin-encoding *E. coli*) (Montero *et al.*, 2017).
653 Hes was shown to promote autoaggregation and biofilm formation as well as
654 erythrocyte agglutination and adherence to epithelial cells, but not invasion. The gene
655 was observed to be present in LEE-negative STEC but not LEE-positive STEC
656 (Montero *et al.*, 2017).

657 1.3.1.3.3. Iron-regulated protein A homologue adhesin (Iha)

658 Iha is an adherent-conferring protein homologous to IrgA (iron-regulated
659 protein A) found in *Vibrio cholerae* (Tarr *et al.*, 2000). As well as a β-barrel structure
660 enabling membrane anchoring as in any OMP, Iha has externally exposed domains.
661 Rather than localised adherence, Iha confers a diffuse adherence pattern in *E. coli*
662 O157:H7. Besides STEC, *iha* has been identified in EPEC and UPEC (Szalo *et al.*,
663 2002, Kanamaru *et al.*, 2003, Gomes *et al.*, 2011). In UPEC, Iha was shown to further
664 act as a catecholate siderophore receptor (Herold *et al.*, 2009) and a virulence factor
665 (Johnson *et al.*, 2005) but these roles in DEC remain to be established. In EHEC, Iha
666 has been clearly demonstrated to be involved in intestinal colonisation and contribute
667 to pathogenesis by promoting adherence to the intestinal epithelium (Yin *et al.*, 2009).

1.3.1.4. Secreted and surface-associated lipoprotein of E. coli (SslE)

SslE, formerly known as YghJ (Yang *et al.*, 2007, Iguchi *et al.*, 2009), was recently described as a novel *E. coli* mucinase thanks to its zinc metallopeptidase motif (Luo *et al.*, 2014, Nesta *et al.*, 2014). This protein is secreted by a Type II, subtype a, secretion system (T2aSS) but the molecular mechanisms of its maturation as a surface lipoprotein remains unclear. The gene encoding SslE is present in different DEC pathotypes such as EPEC, ETEC and EHEC (Decanio *et al.*, 2013). In EPEC, SslE was shown to mediate biofilm formation and intestinal colonisation (Baldi *et al.*, 2012, Vermassen *et al.*, 2019). This protein can be divided into two main variants and antibodies raised against variant I (from ExPEC strain IHE3034) are able to inhibit translocation of *E. coli* strains through a mucin-based matrix. In addition, immunisation of animals with SslE I significantly reduces gut colonisation by strains of different pathotypes expressing SslE II (Nesta *et al.*, 2014). These observations make SslE a key factor in *E. coli* colonisation of the mucosal surface in humans and could serve as a component for a protective vaccine against DEC (Naili *et al.*, 2016, Naili *et al.*, 2017, Rojas-Lopez *et al.*, 2018, Rojas-Lopez *et al.*, 2019).

1.3.1.5. E. coli factor adherence 1 (Efa-1)

Efa-1, also known as LifA (lymphostatin A), present in EPEC and some non-O157 EHEC strains, is known to inhibit the proliferation of mitogen-activated lymphocytes and the synthesis of proinflammatory cytokines, and gamma interferon (Klapproth *et al.*, 2000, Abu-Median *et al.*, 2006). Efa-1 has been shown to mediate colonisation of the calf intestine independently of glycotransferase and cysteine protease motifs (Deacon *et al.*, 2010). In EHEC O157 strains, ToxB is homologous to

Efa-1 and appears to contribute to adherence to cultured epithelial intestinal cells (Tatsuno *et al.*, 2001). However, no lymphostatin-like activity has been associated with this protein and it is not involved in intestinal colonisation in animal models (Stevens *et al.*, 2004, Abu-Median *et al.*, 2006). While Efa-1 has an extracytoplasmic domain and is presumably cell-surface exposed (Nicholls *et al.*, 2002), the molecular mechanisms at play for its secretion and cell-surface display remain unknown.

1.3.1.6. Dispersin

Dispersin is an anti-aggregation protein (Aap) involved in the spreading of bacterial cells along the host intestinal mucosa (Sheikh *et al.*, 2002). This protein contributes to adherence and colonisation of EAEC by preventing hyper-aggregation and collapse of AAF (aggregative adherence fimbriae). Dispersin is present at the bacterial cell-surface *via* binding to LPS in a non-covalent manner after secretion through a Type I secretion system (T1SS) (Velarde *et al.*, 2007). This secretion system and cognate secreted protein are encoded in the *aat* (aggregative ABC transporter) locus located in the pAA plasmid of some EAEC (Nishi *et al.*, 2003). Dispersin is also present in some STEC strains (Monteiro *et al.*, 2009, Muniesa *et al.*, 2012).

1.3.1.7. Moonlighting proteins

At the bacterial cell surface of *E. coli*, some unexpected proteins primarily known to be localised in the cytoplasm have been reported. Among these unexpected cell surface proteins, glycolytic enzymes are frequently uncovered (Henderson & Martin, 2011). These so-called moonlighting proteins have been demonstrated to exhibit a secondary function at the bacterial cell-surface, completely unrelated to their primary function in the cytoplasm (Khan *et al.*, 2014). As a common glycolytic enzyme frequently found at the bacterial cell surface, GAPDH (glyceraldehyde 3-phosphate

716 dehydrogenase) has been demonstrated to bind plasminogen and fibrinogen in EHEC
717 and EPEC (Egea *et al.*, 2007); although there is no evidence of GAPDH acting directly
718 as a plasminogen activator (Coleman & Benach, 1999, Seidler, 2013). In addition,
719 GAPDH is clearly involved in adhesion to intestinal epithelial cells upon infection. A
720 common theme for moonlighting proteins present at the bacterial cell surface is that
721 these proteins lack a N-terminal signal peptide for translocation across the CM and the
722 protein secretion systems enabling their translocation across the OM are often
723 unknown, which is covered by the generic term of non-classical protein secretion
724 (Bendtsen & Wooldridge, 2009, Desvaux *et al.*, 2009). For GAPDH, though, it has been
725 strongly suggested to occur *via* piggybacking through the Type III, subtype a, secretion
726 system (T3aSS) (Aguilera *et al.*, 2012). While it is also known that enolase can also be
727 extracellularly located in *E. coli* (Boel *et al.*, 2004), its contribution to bacterial
728 adhesion remains to be determined. The elongation factor Tu (EF-Tu) is also found at
729 the bacterial cell surface and has been reported to be involved in bacterial aggregation
730 (Amimanan *et al.*, 2017). In DEC, the contribution of putative moonlighting glycolytic
731 enzymes and other moonlighting proteins to the colonisation process deserves more
732 thorough investigation.

733 ### 1.3.2. Multimeric proteinaceous surface colonisation factors

734 Multimeric protein complexes acting as SCFs can be classified as (i)
735 homooligomeric proteins, namely the trimeric autotransporter adhesins (TAAs), and
736 (ii) cell-surface supramolecular structures, including flagella, and numerous pili.

737 #### *1.3.2.1. Trimeric autotransporter adhesins (TAAs)*

738 TAAs are characterised by the presence of a short translocator domain, which
739 is functional upon homotrimeric assembly and corresponds to the Type V, subtype c,

secretion system (T5cSS) (Cotter *et al.*, 2005, Leo *et al.*, 2012). In DEC, TAAs include UpaG (UPEC autotransporter G), Eib (*E. coli* immunoglobulin-binding protein), Sab (STEC-autotransporter mediating biofilm formation) and Saa (STEC autoagglutinating adhesin).

1.3.2.1.1. UPEC autotransporter G (UpaG)

While UpaG was originally identified in UPEC, it was also found in the EAEC 042 strain (Zude *et al.*, 2014). UpaG is involved in autoaggregation, biofilm formation, adhesion to fibronectin, and laminin, as well as human epithelial cells (Valle *et al.*, 2008). In EHEC, EhaG (EHEC autotransporter G) is a positional orthologue of UpaG, which is also involved in autoaggregation, biofilm formation, adhesion to laminin, fibronectin and collagens I, II, II and IV as well as some epithelial cells (Valle *et al.*, 2008, Totsika *et al.*, 2012, Zude *et al.*, 2014). The gene encoding EhaG has been also identified in a wide range of DEC including EPEC, EIEC, ETEC and EAEC (Zude *et al.*, 2014).

1.3.2.1.2. *E. coli* immunoglobulin-binding protein (Eib)

Eibs were originally characterised for their ability to bind immunoglobulin fractions, especially to the Fc (fragment crystallisable) region of IgA and IgG (Sandt & Hill, 2000, Sandt & Hill, 2001, Leo & Goldman, 2009); up to 7 different Eibs have been identified to date, namely EibA, B, C, D, E, F and G. In LEE-negative STEC O91, it further appeared that EibG is involved in adherence to epithelial cells in a chain-like adhesion (CLA) pattern (Lu *et al.*, 2006). CLA corresponds to the formation of a long chain cell aggregate, which EibG induces on both human and bovine intestinal epithelial cells. The gene encoding EibG is distributed into 21 different alleles clustered into three *eibG* subtypes, namely *eibG*-α, -β, and -γ (Merkel *et al.*, 2010). While EibG-α and EibG-β are responsible for the typical CLA phenotype, EibG-γ induces adherence

765 in much shorter cell chains and smaller cell aggregates, corresponding to an atypical
766 CLA. EibD has been further shown to promote autoaggregation and biofilm formation
767 (Leo *et al.*, 2011). Considering their structural similarity, other Eibs have been
768 suggested to have similar biological functions but experimental confirmation is still
769 required to ascertain this. Eib genes are found in some STEC strains, as well as some
770 *E. coli* commensal strains (Lu *et al.*, 2006).

771 1.3.2.1.3. STEC-autotransporter mediating biofilm formation (Sab)

772 Sab contributes to the diffusive adherence of STEC to human epithelial cells
773 and biofilm formation to abiotic surfaces (Herold *et al.*, 2009, Farfan & Torres, 2012).
774 Genes encoding Sab are especially present in LEE-negative STEC.

775 1.3.2.1.4. STEC autoagglutining adhesin (Saa)

776 Saa promotes adhesion to HEp-2 cells in a semilocalised adherence pattern
777 (Paton *et al.*, 2001). So far, the *saa* gene has only been reported in some STEC,
778 including some LEE-negative EHEC strains (Paton & Paton, 2002, Jenkins *et al.*, 2003,
779 Monaghan *et al*., 2011).

780 *1.3.2.2. Cell-surface supramolecular structures*

781 Flagella and pili are organelles resulting from the supramolecular assembly of
782 different protein subunits to form heteromultimeric protein complexes on the bacterial
783 cell-surface.

784 1.3.2.2.1. Flagella

785 Flagellar components are secreted and assembled *via* the Type III, subtype b,
786 secretion system (T3bSS) and more than fifty genes divided in three hierarchical classes
787 are involved in the flagellar apparatus formation (Young *et al.*, 1999, Chilcott &
788 Hughes, 2000). The main component of the flagellum filament is the flagellin, which

789 has considerable diversity in ultrastructure and is responsible for the H-antigen
790 variability (H1 to H56) (Zhou *et al.*, 2015). In *E. coli*, the flagellation is peritrichous
791 but the sites of cell surface localisation and the number of flagella (typically around 6-
792 10) are considered random (Macnab, 1987a, 1987b). Nonetheless, it must be stressed
793 that when swimming, the flagella in motion coalesce into an undulating bundle, forming
794 one rigid helical ponytail about 14 nm in diameter and 10 µm long that appears as
795 polarly localised in *E. coli* (Bray, 2001). A swimming bacterial cell has a run-and-
796 tumble behaviour, where it progresses linearly (run) and then changing abruptly in
797 direction (tumble), but also slow-random-walk behaviour, where it moves at a relatively
798 low speed (Qu *et al.*, 2018). Upon chemotaxis, the rotational direction of the flagella
799 motor can be switched to control motility, a factor that might help approaching the
800 intestinal mucosa in a more coordinated movement (Kitao & Hata, 2018, Rossi *et al.*,
801 2018). The approach to the surface is an important step towards initial bacterial
802 adhesion and subsequent sessile development. Active motility involving the flagella
803 allows the bacterial cells to overcome repulsive electrostatic and hydrodynamic forces
804 at the adhesion site (Donlan, 2002).

805 Besides swimming, flagella can participate in an alternative type of motility
806 called swarming where bacterial cells move and spread on a surface (Kaiser, 2007).
807 Swarming directly contributes to the surface colonisation process and is associated with
808 the expression of an alternative system, the lateral flagella (Merino *et al.*, 2006). In
809 EAEC O42, the Flag-2 locus encodes such a system (Ren *et al.*, 2005), although, a
810 mutation frameshift has likely inactivated this system in this strain. Nonetheless, the
811 Flag-2 cluster appeared to be present in about 20 % of *E. coli* strains from the ECOR
812 collection. In the environmental strain *E. coli* SMS-3-5, although the Flag-2 gene
813 cluster is complete and intact, swarming motility could not be observed (Fricke *et al.*,

2008); to date, the functionality of this system in *E. coli* remains to be elucidated. In the absence of polar flagella, *E. coli* is not as efficient at surface colonisation but is still considered a temperate swarmer, enabling it to swarm over surfaces with rheology corresponding to 0.5 %-0.8 % agar (in comparison to ≥1.5 % agar for robust swarmers) (Partridge & Harshey, 2013).

Besides motility, flagella can directly act as adhesins, as shown in EPEC, where they are involved in adhesion to epithelial cells (Giron *et al.*, 2002, Cleary *et al.*, 2004). In EAEC, flagella contribute to adhesion to plant leaves (Berger *et al.*, 2009). In EHEC, the flagellin FliC favours initial attachment, adhesion to epithelial cells and biofilm formation on abiotic surfaces as well as spinach leaves (McNeilly *et al.*, 2008, Mahajan *et al.*, 2009, Xicohtencatl-Cortes *et al.*, 2009, Vikram *et al.*, 2013, Nagy *et al.*, 2015). In ETEC, flagella contribute to bacterial adhesion to salad leaves and intestinal epithelial cells, as well as biofilm formation (Shaw *et al.*, 2011, Duan *et al.*, 2012, Zhou *et al.*, 2013, Zhou *et al.*, 2014). Interestingly, in this pathotype, flagella can also mediate indirect adhesion through EtpA (ETEC two-partner secretion protein A), a protein secreted by a T5bSS (two-partner secretion system), which bridges the flagella with host cell receptors, thus allowing bacterial cell attachment to some epithelial cells and mucin-expressing regions in mouse small intestine (Fleckenstein *et al.*, 2006, Roy *et al.*, 2009). In EHEC and EPEC, the adhesion of H6 and H7 flagella to the intestinal epithelium and epithelial cells has been suggested to occur though mucins (Giron *et al.*, 2002, Mahajan *et al.*, 2009) as reported for H1 flagella from the probiotic *E. coli* Nissle 1917 (Troge *et al.*, 2012). In some EHEC/STEC strains, namely LEE-negative EHEC O113:H21 and STEC O139:H1:F18ab strains, flagella can also contribute to bacterial invasion of intestinal epithelial cells but the molecular mechanisms at work remains to

838 be clarified (Luck *et al.*, 2006, Rogers *et al.*, 2012, Duan *et al.*, 2013). These latter
839 aspects would undoubtedly deserve further in-depth investigation.

840 While different flagellin variants have been shown to be involved in direct
841 binding to host cells, such as H1 and H19 flagella in ETEC (Duan *et al.*, 2012, Duan *et*
842 *al.*, 2013), systematic analysis of the colonisation properties of all of the different H-
843 antigens in *E. coli* has not been investigated as yet. Except for EIEC which are generally
844 considered as nonmotile (Nataro & Kaper, 1998), the contribution of flagella as a
845 motility factor over an adhesion factor in the colonisation processes has not been clearly
846 resolved as of yet in DEC, particularly regarding bacterial adhesion and biofilm
847 formation to biotic and abiotic surfaces (Wood *et al.*, 2006, Servin, 2014).

848 1.3.2.2.2. Pili

849 Pili, also referred to in *E. coli* literature as fimbriae, are key actors during the
850 initial attachment of bacteria to surfaces, which is characterised by a stronger and longer
851 interaction coupled with a decrease of bacterial motility (Pruss *et al.*, 2006). While
852 binding can be considered reversible as evidenced for the chaperon-usher fimbriae to
853 lectin (Hultgren *et al.*, 1989, Lin *et al.*, 2002), bacterial binding can also be very strong
854 due to the numerous pili expressed simultaneously by a single cell creating an avidity
855 effect, as well as the flexibility of the stalk itself (Andersson *et al.*, 2006). These pili
856 can be secreted and assembled by different protein secretion systems, namely the Type
857 II, subtype c (T2cSS), Type III, subtype a (T3aSS), Type IV, subtype b (T4bSS), Type
858 VII (T7SS) or Type VIII (T8SS) secretion systems (Figure 2). It should be stressed that
859 this numerical protein secretion nomenclature was intended and restricted to the LPS-
860 diderm bacteria in the first place (Desvaux *et al.*, 2009). In mycolate diderm bacteria
861 (archetypical acid-fast bacteria, namely mycobacteria) and some parietal monoderm
862 bacteria, the ESX (ESAT-6) system involved in protein export across the IM (or

cytoplasmic membrane) was also termed T7SS, which is (i) misleading when considering that no ESX component enabling protein translocation across the mycolic outer membrane has yet been identified (Converse & Cox, 2005, Bitter *et al.*, 2009, Groschel *et al.*, 2016, Bosserman & Champion, 2017, Unnikrishnan *et al.*, 2017, Vaziri & Brosch, 2019), and (ii) a misnomer with respect to both the bacterial export systems (and especially parietal monoderm bacteria), which do not follow the numerical nomenclature (e.g. Sec or Tat), and the numerical nomenclature for protein secretion systems in LPS-diderm, which is primarily based on the presence of a translocon at the OM (Desvaux *et al.*, 2004, Desvaux *et al.*, 2009, Desvaux *et al.*, 2009, Sutcliffe, 2011). In diderm bacteria, the ESX is truly an export system in the same line than the Sec or Tat systems (van der Woude *et al.*, 2013) but not a secretion system *per se*. In the present review, the T7SS refers exclusively to the chaperone-usher pathway in LPS-diderm bacteria (Desvaux *et al.*, 2009, Desvaux *et al.*, 2009, Chagnot *et al.*, 2013, Abby *et al.*, 2016, Gagic *et al.*, 2016, Monteiro *et al.*, 2016), which is the main pathway responsible for the secretion of a wealth of pili in *E. coli* (Wurpel *et al.*, 2013). Of note, P pili have been well investigated in UPEC infection (Kuehn *et al.*, 1992, Lillington *et al.*, 2014, Behzadi, 2020) but their prevalence in DEC and potential contribution (or not) in diarrhoeic infection is much less documented although they contribute to intestinal colonisation of commensal *E. coli* (Nowrouzian *et al.*, 2001) and have been detected in some strains causing bovine diarrhoea (Dozois *et al.*, 1997).

1.3.2.2.2.1. The injectisome

The injectisome is a bacterial molecular syringe assembled and secreted by the T3aSS (Desvaux *et al.*, 2006, Galan & Waksman, 2018). The injectisome forms a needle which is functionally closer to the Hrp (hypersensitive response and pathogenicity) pilus in *Pseudomonas syringae* than to a flagellum (He & Jin, 2003,

888 Tampakaki *et al.*, 2004, Cornelis, 2006). This cell-surface appendage can vary in size
889 depending on the bacterial species and even bacterial strains (Cornelis, 2006); in a
890 controlled process, the pilus length can further adapt for cell surface contact. In DEC,
891 this peculiar pilus is encoded by genes located in the LEE pathogenicity island
892 (McDaniel TK, 1995), a landmark for all EPEC but is also present in some EHEC
893 strains (namely the LEE-positive strains), such as *E. coli* O157:H7, and EIEC
894 (including *Shigella* spp.) (Hueck, 1998, Galan & Wolf-Watz, 2006, Coburn *et al.*,
895 2007). Tir (translocated intimin receptor) is encoded by the *tir* gene located in the LEE
896 and is injected in the host cell by the injectisome (Hueck, 1998). This protein is then
897 exposed at the host cell surface and serves as the receptor for the intimin, enabling
898 intimate bacterial interaction with the intestinal epithelia (Donnenberg *et al.*, 1993, Liu
899 *et al.*, 1999). In EPEC, the injectisome is involved in cell adhesion and pedestal
900 formation that occurs during the formation of attaching and effacing lesions upon actin
901 rearrangement in the infected eukaryotic cell (A/E) (Wong *et al.*, 2011). Of note, while
902 A/E lesions are observed *in vitro* from infected epithelial cell cultures or colonic
903 epithelium with LEE-positive EHEC (Lewis *et al.*, 2015), these kinds of lesions are
904 never observed from clinical samples of EHEC infections (Nataro & Kaper, 1998); a
905 clear explanation of why this is the case is unclear but would undoubtedly deserve
906 further investigation to match up lab experiments with clinical observations (Lewis *et*
907 *al.*, 2015). In addition to the infection of mammalian cells, the injectisome is involved
908 in adhesion to plants with a marked tropism for the stomata (Schroeder & Hilbi, 2008,
909 Shaw *et al.*, 2008, Berger CN, 2010, Croxen *et al.*, 2013). EspA, the main component
910 of the filament in the injectisome is directly involved in adhesion, as well as in biofilm
911 formation, in EPEC (Knutton *et al.*, 1998, Moreira *et al.*, 2006). In EIEC, the
912 injectisome contributes to the invasion capabilities (Hueck, 1998).

913 *1.3.2.2.2.2. Type 4 pili (T4P)*

914 T4P are assembled and secreted by the T2cSS (Ramer *et al.*, 2002, Chagnot *et*
915 *al.*, 2013). T4P have been demonstrated to play a role in several *E. coli* pathotypes,
916 including host cell adherence and bacterial aggregation (Craig *et al.*, 2004). Some of
917 these pili can exhibit a unique feature in their ability to extend and retract, which results
918 in twitching motility further contributing to biofilm formation (Mattick, 2002, Craig *et*
919 *al.*, 2019). In EPEC, T4P are also known as BFP (bundle-forming pili) and their
920 subunits assemble in a helical manner to form polymeric fibres and can further interact
921 to create higher-order bundles or tangled aggregates (Giltner *et al.*, 2012, Melville &
922 Craig, 2013). These T4P are involved in the colonisation of the GIT and contribute to
923 bacterial virulence (Bieber *et al.*, 1998, Tacket *et al.*, 1998). BFP are encoded by the
924 *bfp* operon comprising of 14 genes, including *bfpA*, which encodes the major repeating
925 subunit of the pilus fibre (Ramer *et al.*, 1996, Sohel *et al.*, 1996). In EHEC strains, the
926 T4P are called HCP (haemorrhagic *E. coli* pili) (Xicohtencatl-Cortes *et al.*, 2009).
927 Inactivation of the *hcpA* gene in EHEC O157:H7 reduces adherence to human and
928 bovine epithelial cells. HCP is also able to bind to fibronectin and laminin, to
929 agglutinate rabbit red blood cells, to mediate biofilm formation and to promote
930 twitching motility (Xicohtencatl-Cortes *et al.*, 2009). HCP are also encoded in some
931 STEC strains (Farfan & Torres, 2012). Because of their size, peculiar T4P called longus
932 pili have been reported in ETEC (Giron *et al.*, 1994). The N-terminal part of the major
933 subunit LngA is homologous with Bfp of EPEC, CofA subunit of CFA/III (colonisation
934 factor antigen) of ETEC and TCP (the toxin-coregulated pilin) of *V. cholerae* (Giron *et*
935 *al.*, 1995, Gomez-Duarte & Kaper, 1995). Longus pili are involved in colonisation of
936 the human gut (Clavijo *et al.*, 2010, Mazariego-Espinosa *et al.*, 2010), in bacterium-

bacterium interaction and resistance to antimicrobial agents as a result of biofilm formation (Clavijo *et al.*, 2010).

1.3.2.2.2.3. Conjugative pili (CP)

CP are assembled and secreted through T4bSS (Lawley *et al.*, 2003). Classically, the genes encoding for F-plasmid transfer are encoded on the *tra* operon located in the conjugative F plasmid (Manwaring *et al.*, 1999). CP are responsible for nucleoprotein transfer between a donor bacterial cell (harbouring the F plasmid) and a recipient bacterial cell *via* the T4bSS (Lawley *et al.*, 2003). Bacterial conjugation is a well-known process enabling horizontal transfer of genes including virulence or colonisation factors (Manwaring *et al.*, 1999, Mazel & Davies, 1999, Llosa *et al.*, 2002, Sorensen & Mortensen, 2005). Gene transfer is especially promoted in biofilm where physical contact between sessile donor and recipient cells is favoured (Lebaron *et al.*, 1997, Hausner & Wuertz, 1999, Dionisio *et al.*, 2002, Molin & Tolker-Nielsen, 2003, Maeda *et al.*, 2006). Besides the transfer of genetic material, CP can be directly involved in bacterial adhesion (Beloin *et al.*, 2008, May & Okabe, 2008, May *et al.*, 2011). In biofilm, this can be further amplified as cells carrying a conjugative F plasmid promote the establishment of F pili mating pairs and consequently induce adhesion and biofilm formation between abiotic surfaces and poor biofilm former cells. EAEC strains expressing F pili have been demonstrated to improve mixed biofilm formation (Pereira *et al.*, 2010). In EAEC C1096, pili encoded on the conjugative plasmid IncI1 further contributed to adherence to abiotic surfaces and epithelial cells (Dudley *et al.*, 2006). In EHEC O157:H7 Xuzhou, a novel conjugative plasmid called pO157-Sal encoding a complete set of genes for the T4bSS was identified, but its involvement in the colonisation process has not been investigated as yet (Wang *et al.*, 2011, Zhao *et al.*, 2013).

1.3.2.2.2.4. Type 1 pili (T1P)

T1P (also called Type 1 fimbriae) are the most investigated pili secreted and assembled *via* a T7SS (Capitani *et al.*, 2006). The expression of T1P is induced during the initial bacterial adhesion step (Harris *et al.*, 1990, Pratt & Kolter, 1998, Cookson *et al.*, 2002, Orndorff *et al.*, 2004, Reisner *et al.*, 2014) and they are involved in the early and late stages of biofilm formation (Schembri *et al.*, 2003, Beloin *et al.*, 2004, Reisner *et al.*, 2014). T1P also have a role in the formation of SIgA (secretory IgA) mediated biofilm of the normal flora within the gut (Bollinger *et al.*, 2003, Orndorff *et al.*, 2004, Bollinger *et al.*, 2006). T1P are composed of FimA (fimbrillin A), which constitutes the pilus rod, and FimH at the apex of the pilus tip. FimH is the key adhesin component in T1P as it can link to mannose residues of some receptors on eukaryotic cells (Kaper *et al.*, 2004, Duncan *et al.*, 2005) but also has nonspecific binding activity to abiotic surfaces (Pratt & Kolter, 1998, Beloin *et al.*, 2008). The absence of the FimH adhesin has been shown to hinder biofilm formation by preventing cell-to-surface and cell-to-cell contacts (Danese *et al.*, 2000). In *E. coli*, different *fimH* alleles have been reported as conferring distinct colonisation abilities and thus playing different roles in biofilm formation (Martinez *et al.*, 2000, Weissman *et al.*, 2006). It was shown that contact between T1P and abiotic surfaces alters the composition of the OM and changes some physicochemical properties of the bacterial surface, which in turn influences adhesion (Otto *et al.*, 2001, Orndorff *et al.*, 2004). While the laboratory *E. coli* K12 strain and UPEC NU14 strain are the focus of the majority of the investigations about T1P, their involvement in bacterial adhesion and/or biofilm formation has been further demonstrated in EPEC, EAEC, ETEC and STEC strains (Elliott & Kaper, 1997, Cookson *et al.*, 2002, Moreira *et al.*, 2003, Sheikh *et al.*, 2017). T1P are encoded in the *fimBEAICDGHF* gene cluster, which is quite widespread in *E. coli* in both commensal

and pathogenic isolates (Sauer *et al.*, 2000, Kaper *et al.*, 2004, Wurpel *et al.*, 2013). While present in EHEC O157:H7 (Abraham *et al.*, 1988, Li *et al.*, 1997, Roe *et al.*, 2001, McWilliams & Torres, 2014), their contribution to the colonisation process has yet to be demonstrated.

Genes encoding the F1C pili are present in approximately 7 % of *E. coli* faecal isolates (Werneburg & Thanassi, 2018). F1C pili have been characterised in UPEC strains where they are encoded in the *foc* (fimbriae of serotype 1C) operon homologous to the *fim* locus (Klemm *et al.*, 1994). In UPEC, F1C pili are involved in adherence to the bladder and kidney cells, as well as in biofilm formation (Werneburg & Thanassi, 2018). Their prevalence and contribution to the colonisation process in DEC remains to be investigated.

1.3.2.2.2.5. CS31A pili

The CS31A (coli surface associated 31a antigen) plays a key role in the virulence of septicemic *E. coli* and ETEC, as well as some EPEC and DAEC (Girardeau *et al.*, 1988, Contrepois *et al.*, 1989, Jallat *et al.*, 1994, Adams *et al.*, 1997). Because of their thin structure, as well as their close and packed association to the bacterial cell surface, CS31A was initially described as capsule-like or even nonfimbrial antigens (Bertin *et al.*, 1993, Mechin *et al.*, 1996) before being clearly identified as thin capsular pili secreted and assembled by a chaperone-usher pathway (T7SS) (Thanassi *et al.*, 1998). These pili are synthesised from the *clp* operon located on a high-molecular-weight self-transmissible R plasmid, called p31A (Martin *et al.*, 1991, Jallat *et al.*, 1994, Martin, 1996). CS31A are considered homologous to the K88/F4 (*fae* operon) and F41 pili but with some functional dissimilarities, such as that CS31A does not exhibit haemagglutinin activity (Girardeau *et al.*, 1991). In ETEC, F4 pili allow bacterial adherence to F4-specific receptors present on the brush borders of villous enterocytes

thus promoting the colonisation of the small intestine (Snoeck *et al.*, 2008). The locus for diffuse adherence (*ldaCDEFGHI*) (Scaletsky *et al.*, 2005) from EPEC is homologous to the K88 *fae* and ETEC CS31A *clp* operons. LdaH mediates diffuse adherence to Hep-2 cells. The LdaH encoding gene has also been found in STEC strains but no functional characterisation has been reported as yet (Scaletsky *et al.*, 2005).

1.3.2.2.2.6. Aggregative adherence fimbriae (AAF)

AAF belongs to the Afa/Dr (afimbrial adhesin/decay-accelerating factor receptor) haemagglutinin family together with F1845 pili (Nowicki *et al.*, 1990, Le Bouguenec & Servin, 2006). In DAEC and EIEC, Afa and Dr hemagglutinins recognise the Dr blood group antigen (Nowicki *et al.*, 1990). Among the five genes encoded in the *afa* cluster, *afaB*, *afaC* and *afaE* are required for mannose-resistant hemagglutination (MRHA) (Servin, 2005). The Dr hemagglutinin is encoded by the *draABCDE* operon, where *draA*, *draB*, *draC*, and *draD* encode accessory proteins and *draE* encodes the adhesin part (Nowicki *et al.*, 1987, Servin, 2005). In addition, it specifically binds collagen IV (Nowicki *et al.*, 1988). Afa and Dr haemagglutinins can link to decay-accelerating factor (DAF) and to carcinoembryonic antigen-related cellular adhesion molecules (CEACAMs) (Nowicki *et al.*, 1988, Westerlund *et al.*, 1989, Berger *et al.*, 2004). While some members of the Afa/Dr family were believed not to form pili as they could not be observed by electron microscopy examination, it is now clear they are secreted as AAF and F1845 by T7SS, to form pili of various architecture depending on the pilin subunits (Anderson *et al.*, 2004, Pettigrew *et al.*, 2004).

In EAEC, the colonisation of the gut occurs through aggregative adherence (AA) due to AAF, which binds to ECM proteins such as fibronectin, laminin and collagen IV (Farfan *et al.*, 2008, Berry *et al.*, 2014) and then promotes biofilm

formation (Hicks *et al.*, 1996, Wakimoto *et al.*, 2004). To date, five AAFs (AAF/I to AAF/V) have been identified, all encoded by virulence plasmids of EAEC (pAA) and the main subunits of which are AggA, AafA, Agg3A, Agg4a and Agg5a respectively (Nataro *et al.*, 1992, Czeczulin *et al.*, 1997, Boisen *et al.*, 2008, Jonsson *et al.*, 2015). Another hypothetical Dr-related pilin called HdaA (HUS-associated diffuse adherence) also appears to confer the capacity to cause the AA phenotype in EAEC (Boisen *et al.*, 2008). In DAEC and EIEC, F1845 pili are involved in gut colonisation (Servin, 2005). F1845 pili are responsible for diffuse adherence to epithelial cells of the gut and are encoded by the *daaABCDE* operon (Bilge *et al.*, 1989, Bilge *et al.*, 1993).

1.3.2.2.2.7. Colonisation factor antigens (CFA)

In ETEC, colonisation factor antigens (CFA), also called coli surface antigens (CS), form pili that take part in adhesion to the small intestine and are critical for virulence (Gaastra & Svennerholm, 1996). CFA/I, CFA/II (CS1, 2 and 3) and CFA/IV (CS4, 5 and 6) are the most virulent (Sjoberg *et al.*, 1988, Knutton *et al.*, 1989, Taniguchi *et al.*, 1995, Gaastra & Svennerholm, 1996, Svennerholm & Lundgren, 2012) but CS12, 14, 17, 18, 19, 20 and 31 can also adhere to intestinal cells (Werneburg & Thanassi, 2018). CFA/CS are encoded in operons; taking CFA/I as an example, it is encoded by the *cfaABCE* operon, where *cfaB* encodes the main subunit, *cfaE* the distal subunit, *cfaA* a chaperone and *cfaC* the usher involved in pilin transport across the OM (Jordi *et al.*, 1992). Cell adhesion is enabled by CfaB through its ability to bind glycosphingolipid (Jansson *et al.*, 2006).

1.3.2.2.2.8. F9 pili

In EHEC O157:H7, F9 pili are involved in the colonisation of epithelial bovine cells, bovine gastrointestinal tissue explants and can also bind to fibronectin (Low *et al.*, 2006). Mutants of the main subunit of F9 pili are still able to colonise the terminal

rectum, indicating that the adhesin is not solely responsible for the rectal tropism observed but may contribute to colonisation at other sites, especially in young animals (Low *et al.*, 2006). These pili are short but are able to form longer bundles (Low *et al.*, 2006). They are encoded in the F9 gene cluster, a six genes operon located on the pathogenicity island O161 (Low *et al.*, 2006, Wurpel *et al.*, 2013). This operon has also been identified in EPEC, as well as EAEC (Wurpel *et al.*, 2013). F9 pili are secreted and assembled by a T7SS (Wurpel *et al.*, 2013).

1.3.2.2.2.9. E. coli YcbQ laminin-binding fimbriae (ELF)

In EHEC O157:H7, it has been shown that *E. coli* YcbQ laminin-binding fimbriae (ELF) bind laminin and are involved in adherence to epithelial cells in humans, cows and pigs (Samadder *et al.*, 2009). ELF form peritrichous flexible fine fibres and are encoded by the *elfADCG* operon, originally called the *ycbQRST* operon, which was previously identified in UPEC and some commensal *E. coli* strains (Spurbeck *et al.*, 2011). This operon is homologous to the F17 pili biogenesis genes found in ETEC, which are assembled and secreted by a T7SS (Lintermans *et al.*, 1988, Lintermans *et al.*, 1991, Bertin *et al.*, 1996, Bertin *et al.*, 2000). More generally, ELF are also homologous to 20K, K99 and G pili found in various pathogenic *E. coli* (Guinee *et al.*, 1976, Contrepois *et al.*, 1983). These pili have been shown to mediate binding to intestinal mucosal cells, especially to N-acetyl-D-glucosamine-containing receptors (Bertin *et al.*, 1996). The composition of the pili and the sequence of the tip-adhesin differ between the strains and could explain the phenotypic divergence associated with the expression of this family of pili in different *E. coli* strains (Korea *et al.*, 2010).

1.3.2.2.2.10. Long polar fimbriae (LPF)

LPF are encoded by two operons *lpf1* and *lpf2* located on the pathogenicity islands O141 and O154 in EHEC O157:H7, respectively (Perna *et al.*, 2001). LPF are

also present in other DEC, e.g. LEE-negative EHEC, EPEC, rabbit-specific EPEC, EAEC and ETEC, as well as in several commensal strains (Doughty *et al.*, 2002, Wurpel *et al.*, 2013). They share homology with the LPF of *Salmonella enterica* serovar Typhimurium which are involved in adherence to Peyer's patches and M cells in the human gut (Baumler & Heffron, 1995, Baumler *et al.*, 1996). The *lpf1* operon is composed of five genes, with *lpfA* encoding the main pilus subunit, *lpfD* and *lpfE* encoding minor subunits, and *lpfB* and *lpfC* encoding the chaperone and usher respectively (Doughty *et al.*, 2002, Torres *et al.*, 2004). The *lpf2* operon also contains five genes with a duplication of *lpfD* called *lpfD'* but with no *lpfE* paralogue (Torres *et al.*, 2004). In *E. coli* O157:H7, it has been proposed that LPF2 is expressed in early stages whereas LPF1 is expressed in late stages of growth (Torres *et al.*, 2004). LPF are secreted and assembled by a T7SS and can bind fibronectin, laminin and collagen IV, as well as the follicule-associated epithelium (FAE) of Peyer's patches in humans (Fitzhenry *et al.*, 2006, Farfan & Torres, 2012, McWilliams & Torres, 2014). Expression of *lpf2* is increased under conditions similar to those for biofilm formation (Torres *et al.*, 2007). Recently, it has been demonstrated that STEC isolates positive for *lpf2* formed significantly more biofilm than *lpf2*-negatives isolates (Vogeleer *et al.*, 2015). In EPEC, LPF have been shown to contribute to the early stages of colonisation of rabbits and the severity of diarrhoea (Newton *et al.*, 2004).

1.3.2.2.2.11. E. coli common pilus (ECP)

In EHEC, ECP (previously called Mat for meningitis-associated temperature dependent pilus) provides adherence to HEp-2, HeLa and HT-29 cells and allows interaction between bacterial cells (Rendon *et al.*, 2007). Secreted and assembled by a T7SS, ECP expression is increased under environmental conditions that are experienced in the GIT, *e.g.* low oxygen and high CO_2 concentrations (Rendon *et al.*,

2007). However, its role seems to be secondary in the colonisation of the human or bovine gut (Tatsuno *et al.*, 2000, Dziva *et al.*, 2004). The *ecp* operon has been identified in numerous commensal and pathogenic *E. coli*, including DEC (Rendon *et al.*, 2007).

1.3.2.2.2.12. Sorbitol-fermenting frimbriae protein (SFP)

In EHEC, the expression of sorbitol-fermenting frimbriae protein (SFP) pili is induced in anaerobic conditions and leads to an increased adherence to Caco-2 and HCT-8 cells, with a mannose-resistance hemagglutination phenotype (Brunder *et al.*, 2001, Musken *et al.*, 2008, Bielaszewska *et al.*, 2009). These pili are encoded on the *sfpABDCDJG* operon harboured in the virulence plasmid pSFO157 (Brunder *et al.*, 2006). SFP pili are secreted and assembled by a T7SS (Brunder *et al.*, 2001). Besides *E. coli* O157, *sfp* has been identified in other EHEC serotypes, such as O165 (Bielaszewska *et al.*, 2009), but its prevalence among STEC in general is thought to be quite low (Toma *et al.*, 2004). Distribution of the *sfp* operon in other DEC has not been investigated in detail as of yet.

1.3.2.2.2.13. Curli

Curli are thin aggregative pili generally considered as one of the major proteinaceous components of the *E. coli* biofilm matrix (Smyth *et al.*, 1996, Stathopoulos *et al.*, 2000, Kostakioti *et al.*, 2005, Evans & Chapman, 2014). These peculiar pili are secreted and assembled by the T8SS through the extracellular-nucleation-pathway (ENP). Curli are helical filamentous amyloid fibres that facilitate cell-surface and cell-cell interactions and promote biofilm formation (Olsen *et al.*, 1993, Cookson *et al.*, 2002, Szabo *et al.*, 2005, Beloin *et al.*, 2008, McCrate *et al.*, 2013). In EHEC O157:H7, curli are associated with cellulose production, adherence to spinach leaves and Hep-2 cells as well as abiotic surfaces (Kim & Kim, 2004, Pawar *et al.*, 2005, Macarisin *et al.*, 2012). In ETEC, curli facilitate adherence to plastic surfaces

(Szabo *et al.*, 2005). Although curli were originally thought not be expressed by EPEC (Ben Nasr *et al.*, 1996), some strains were later reported to synthetise curli, playing a role in bacterial adhesion and biofilm formation in condition mimicking human or bovine hosts (Saldana *et al.*, 2009). However, curli do not seem to be required for biofilm formation and/or adhesion of EAEC strains (Sheikh *et al.*, 2001, Berger *et al.*, 2009, Pereira *et al.*, 2010). In *Shigella spp*. and EIEC, CsgD and curli expression is often inactivated (Sakellaris *et al.*, 2000). Two operons are involved in curli production, (i) the *csgBAC* operon, encoding the structural components of curli (CsgA and CsgB) and an accessory protein (CsgC), and (ii) the *csgDEFG* operon, encoding a transcriptional regulator (CsgD) and the secretion machinery for transport across the OM (CsgE-G) (Arnqvist *et al.*, 1994, Hammar *et al.*, 1995, Beloin *et al.*, 2008). In the current model, CsgB is proposed as embedded in the OM where it acts as a nucleator for the polymerisation of the major CsgA curlin (Van Gerven *et al.*, 2015, Jain & Chapman, 2019). While the exact structure of curli fibres has not yet been elucidated with molecular resolution (Van Gerven *et al.*, 2015, Jain & Chapman, 2019), the fibres have been reported to display irregular thin branches, which would result from minor incorporation of CsgB along the curli and promoting the formation of branched fibres (Bian & Normark, 1997, Soto & Hultgren, 1999, Shu *et al.*, 2012, DeBenedictis *et al.*, 2017). Recently, CsgC and CsgE were demonstrated to highly inhibit CsgA aggregation and CsgE was shown to prevent pellicle biofilm formation when added exogenously (Andersson *et al.*, 2013, Evans *et al.*, 2015).

1.3.2.2.2.14. Haemolysin-coregulated protein (Hcp)

In EAEC, the haemolysin-coregulated protein (Hcp) tube formed by the Type VI secretion system (T6SS) was suggested to be of importance for biofilm formation (Aschtgen *et al.*, 2008). More than ten orthologues of the T6SS components have been

identified in EHEC and EPEC strains. This system can also contribute to bacterial aggregation at the host cell surface (Dudley *et al.*, 2006, Shrivastava & Mande, 2008, Lloyd *et al.*, 2009, Aschtgen *et al.*, 2010, Moriel *et al.*, 2010). Further investigations are required in DEC to determine the exact role and molecular mechanisms involved in the colonisation processes by the Hcp and T6SS.

2. The different regulation levels involved in the expression of colonisation factors

In general, the expression of genes encoded on genomes into proteins can be regulated at pre-transcriptional, transcriptional, post-transcriptional, translational and/or post-translational levels, as well as at translocational and post-translocational levels, the latter of which are especially relevant and important for molecular determinants expressed at the bacterial cell surface (Figure 3). With the rise of omic approaches, however, some basic bacterial physiology concepts may sometimes be overlooked and gene/protein expression is very often considered as being limited to regulatory networks involving transcriptional repressors or activators. However, when it comes to functions and activities, it is primarily proteins that can help to comprehend bacterial physiology. It must also be kept in mind that the relationship between mRNA and protein abundances only very partially correlates; mRNA levels are just a proxy for the presence of a protein but is not directly proportionate with the increase or decrease folds of protein expression and even less with its activity when we consider an enzyme for instance (Vogel & Marcotte, 2012). Here, the different regulatory levels involved in bacterial adhesion and biofilm formation are highlighted using key examples of different SCFs.

2.1. Regulation at the pre-transcriptional level: phase variation

Prior to transcription, some regulatory mechanisms can already be at work at the DNA level, through phase variation. There are four main mechanisms of phase variation (i) DNA inversion, (ii) slipped-strand mispairing, (iii) DNA methylation, and (iv) DNA deletion (Henderson *et al.*, 1999). As a commonality, all these regulatory mechanisms primarily occur at the stage of DNA replication and a large majority of genes regulated by phase variation are bacterial cell surface molecular determinants (Owen *et al.*, 1996, Holden & Gally, 2004).

In *E. coli* K12, T1P are well-known to be subjected to phase variation following DNA inversion (Blomfield, 2001). The expression of the *fim* operon is under the control of the *fim* promoter, which is located within the *fimS*-invertible element (Abraham *et al.*, 1985, Wright *et al.*, 2007). The orientation of the promoter determines the ON or OFF phase and then induces the expression of upstream genes or not. Two tyrosine recombinases, FimB and FimE, are known to control the orientation of the *fimS*-invertible region. FimB predominantly switches the *fim* operon transcription from OFF to ON, while FimE mediates ON to OFF phase switching (Klemm, 1986, Gally *et al.*, 1996, Hannan *et al.*, 2008). Of note, two DNA topological effectors participate in this regulation, namely H-NS (histone-like nucleoid-structuring protein) and IHF (integration host factor); these histones play complementary role, as the DNA inversion is absolutely dependent upon IHF, whereas the inversion rate is slowed down with high levels of H-NS and *vice versa* (Dorman & Ni Bhriain, 1993). The existence of this regulation in DEC has not been examined as of yet.

Slipped-strand mispairing occurs in the course of DNA replication in repetitive DNA regions, which can be positioned either upstream of a coding DNA sequence

(CDS) and then influences the transcription, such as the promoter efficiency, or within a CDS and can affect the translational reading frame resulting in a mutation frameshift (Henderson *et al.*, 1999). In *E. coli*, phase variation resulting from strand-slippage has not been reported as yet, nonetheless, there is no molecular mechanistic constraint for it not to occur (Torres-Cruz & van der Woude, 2003).

Phase variation resulting from DNA methylation corresponds to a bacterial epigenetic mechanism (Henderson *et al.*, 1999). Ag43 is probably one of most investigated surface proteins subjected to such a regulatory mechanisms (van der Woude & Henderson, 2008). This epigenetic regulation involves two proteins, the DNA adenine methylase (Dam) and the OxyR transcriptional regulator (van der Woude & Henderson, 2008). When Dam has methylated the GATC sites present in the operator region in the course of DNA replication, the repressor OxyR cannot bind and transcription by the RNA polymerase occurs and Ag43 is expressed (ON phase); however, if OxyR binds the GATC sites before they are methylated by Dam, there is no transcription and no Ag43 expression (OFF phase). Besides Ag43, several pili secreted and assembled by the T7SS have been reported to be subjected to such an epigenetic regulation in *E. coli* (Henderson *et al.*, 1999, Blomfield, 2001). The *pap* (pyelonephritis-associated pilus) operon in UPEC is considered as a paradigm where the Dam methylation of a GATC-II site in the operator region prevents binding of the repressor Lrp (leucine-responsive regulatory protein), and consequently the *papBA* operon is transcribed and the pili are expressed (ON phase). In the absence of methylation at GATC-II, Lrp can bind to the operator, repress the transcription and ultimately prevent pili formation (OFF phase). Additionally, this repression can be lifted when Lrp binds to another site called GATC-I. Among DEC, CS31A pili are subjected to this same regulatory mechanism (Crost *et al.*, 2003, Graveline *et al.*, 2014).

As a general trend, phase variation due to DNA deletion is irreversible due to the loss of the genetic element bearing the gene of interest. In *E. coli*, DNA deletion is responsible for unilateral flagellar phase variation as reported in the H3, H47 and H17 strains (Zhou *et al.*, 2015). While most flagellins are encoded by *fliC* in *E. coli*, H3 and H47 are encoded by *flkA* and H17 is encoded by *flnA*. For H3 and H47, their production results from the expression of *flkAB* operon, where the transcriptional regulator FlkB represses *fliC* (Feng *et al.*, 2008). Upon excision of the *flk* region from the chromosome, *flkAB* is irreversibly deleted, the repression of *fliC* is released and the FliC flagellin is produced. Similarly, the H17 strain can irreversibly switch flagellar antigens to H4 (Ratiner, 1967). It appears this flagellar phase variation can be caused by excision of *flnA* (Liu *et al.*, 2012). When *flnA* is present in the chromosome, the translation of FliC H4 is inhibited and only FlnA H17 is produced; once *flnA* is excised, the repression of the *fliC* is released and only the FliC H4 is produced. The ~35 kb DNA deletion region containing the *flnA* gene is excised as a covalently closed extrachromosomal circular form. While some DNA deletion can occur through homologous recombination (Henderson *et al.*, 1999), flagellar phase variation is mediated by non-homologous recombination *via* an integrase of the tyrosine recombinase family (Feng *et al.*, 2008). The flagellar phase variation mechanisms in some other *E. coli* H variants and especially in DEC remain to be defined.

2.2. Regulation at the transcriptional level: regulators and effectors

Regulation at the transcriptional level is the most well-known level of gene regulation and quite often the only one really considered as a proxy for protein expression levels. Transcriptional regulators can either be repressors or activators but

it is wrong to assume a repressor will systematically repress transcription or an activator will activate transcription. A second crucial partner to the process must also be considered, that is the effector, which can be of two types, either an inducer or a co-repressor. Four possibilities for regulation at the transcriptional level can be discriminated: (i) positive control of an inducible gene, where an activator is activated by an inducer, (ii) positive control of a repressible gene, where an activator is inactivated by an inhibitor, (iii) negative control of an inducible gene, where a repressor is inactivated by an inducer, or (iv) negative control of a repressible gene, where a repressor is activated by a co-repressor. Additionally, a so-called repressor can act as an activator for some genes and vice versa. In other words, the up-expression or down-expression of a regulator is not sufficient to know what kind of transcriptional regulation is taking place without knowing the nature and level of the inducer.

Bacteria can sense and respond to environmental cues thanks to a large range of two-component signal transduction systems where a sensor activates a transcriptional regulator, which further represses or activates gene expression (Hoch, 2000, Zschiedrich *et al.*, 2016). Some of these systems participate in cell-to-cell communication (CTCC) *via* a signal molecule called auto-inducer (AI) (Bassler, 2002). Quorum sensing (QS) is only one of the different functions of CTCC, which specifically refers to the sensing of the cell density (quorum); QS should not be considered synonymous with CTCC because some sensing can be unrelated to QS *sensu stricto* but to diffusion sensing, confinement or efficiency sensing for instance (Redfield, 2002, Platt & Fuqua, 2010, West *et al.*, 2012). This semantic issue is of particular importance in biofilm formation, since by definition, bacteria cells are at a high density following sessile development and therefore the notion of QS makes little sense. Transcriptional regulators of virulence and SCFs have been the subject of intense and extensive

research and scientific literature in DEC (Beloin *et al.*, 2008, Tobe, 2008, Pruss, 2017, Rossi *et al.*, 2018). For these reasons only some key examples will be provided to illustrate the relevance of differentiating the regulation at different levels.

At the transcriptional level, PNAG production is regulated by NhaR, a transcriptional regulator of the LysR family, which activates the transcription of the *pgaABCD* operon by binding to two sites near the -35 region of the promoter (Goller *et al.*, 2006). In EPS, the production of colanic acid is consistently upregulated within biofilms by the RcsA transcriptional activator (Matthysse *et al.*, 2008, May & Okabe, 2008). The transcription of the *wca* operon is regulated by the *rcsABCF* locus that encodes a two-component system (Gervais & Drapeau, 1992, Ebel & Trempy, 1999, Beloin *et al.*, 2008). However, the signal sensed by the RcsC sensor kinase remains unknown (Whitfield & Roberts, 1999, Oropeza *et al.*, 2015). H-NS is known to act as a transcriptional repressor in bacteria, a so-called bacterial transcriptional silencing, analogous to eukaryotic silencing by histones (Landick *et al.*, 2015, Grainger, 2016). While RcsA is present at a low amount in the cell, this was found to be partially due to transcriptional silencing by H-NS (Sledjeski & Gottesman, 1995). Cellulose synthesis is under the control of the CsgD transcriptional regulator (Romling *et al.*, 2000, Zorraquino *et al.*, 2013). Interestingly in EIEC, *csgD* expression is often inactivated (Sakellaris *et al.*, 2000), suggesting that biofilm formation can interfere with pathogenesis, making these strains poor biofilm formers.

While no specific transcriptional regulator has been identified for the expression of AIDA-I, it was shown that transcription was enhanced in the absence of H-NS and RfaH transcriptional regulators (Benz *et al.*, 2010). Similarly, the transcription of *ehaG* and *fdeC* is regulated by H-NS (Totsika *et al.*, 2012, Easton *et al.*, 2014).

CS31A synthesis is dramatically reduced in media containing alanine or leucine, suggesting that these amino acids can play a role as effectors (Crost *et al.*, 2003). The ON/OFF switch is locked in the OFF phase by alanine, whilst leucine repressed transcription but without affecting the switch frequency. Analysis of *clp* expression indicated that alanine and leucine could repress *clp* transcription by a methylation-independent mechanism but also by either promoting methylation or methylation protection of GATC-II and GATC-I respectively, which increased the methylation pattern characteristic of repressed cells. Furthermore, alanine prevented the AfaF-dependent methylation protection and thus the appearance of cells in the ON phase. Additional regulatory proteins, including ClpB, cAMP, receptor protein (CRP) and H-NS, also play important roles in the transcriptional expression of the operons of the *pap* family combined with regulation at a pre-transcriptional level by phase variation (Blomfield & van der Woude, 2007).

For the T4P in EPEC, the expression of the *bfp* operon is controlled by the BfpT (also called PerA) transcriptional regulator, a member of the AraC family, encoded on the enteroadherence factor plasmid (Tobe *et al.*, 1992, Gomez-Duarte & Kaper, 1995). The expression of CFA/I is positively regulated by CfaR, whereas for the expression of CFA/II, CS1 and CS2 is positively regulated by the *rns* gene product (a homologue to *cfaR* with 96 % identity) (Caron & Meyer, 1989, Caron & Scott, 1990, Savelkoul *et al.*, 1990). The expression of AAF is induced by the transcriptional activator AggR (an homologue of AraC) also located on pAA (Nataro *et al.*, 1994); YafK and Fis (factor for inversion stimulation) have also been reported to regulate AAF/II transcription (Sheikh *et al.*, 2001). From a transcriptional regulation point of view, *lpf1* is repressed by H-NS and activated by Ler in response to different environmental conditions (Torres *et al.*, 2007, Rojas-Lopez *et al.*, 2011), whereas *lpf2* transcription appears to be

activated by Fur (Torres *et al.*, 2007). Regulation of curli biogenesis is complex and involves several two-component systems, such as EnvZ/OmpR, CpxA/CpxR or CpxR/H-NS/RstA/IHF/OmpR (Vidal *et al.*, 1998, Prigent-Combaret *et al.*, 2000, Prigent-Combaret *et al.*, 2001, Beloin *et al.*, 2008, Ogasawara *et al.*, 2010, Laverty *et al.*, 2014). In EPEC, Fis has been identified as a negative transcriptional regulator of *csgA* expression (Saldana *et al.*, 2009). Curli expression can be triggered by a large range of environmental signals such as the temperature, osmolarity or redox potential (Olsen *et al.*, 1993, Prigent-Combaret *et al.*, 1999, Gerstel & Romling, 2001, Evans & Chapman, 2014).

The transcriptional regulatory control of the locus of enterocyte effacement (LEE) encoding the injectisome is undoubtedly one of the most extensively investigated in DEC, and in particular in EPEC and EHEC (Schmidt, 2010, Stevens & Frankel, 2014, Franzin & Sircili, 2015). For additional information about the complex regulation networks of specific, global and phage encoded regulators, as well as environmental signals such as nutrient sources or metabolic products from the host or microbiota that can affect the transcription of the LEE-encoded genes, readers are referred to recent, specific reviews on the topic (Connolly *et al.*, 2015, Furniss & Clements, 2018, Platenkamp & Mellies, 2018, Turner *et al.*, 2018).

2.3. Regulation at a post-transcriptional level

At least three main regulation mechanisms can occur post-transcriptionally, (i) the stability of mRNA, which can be quantified by determining its half-life, (ii) a riboswitch, where a molecule such as a metabolite can change the folding of an mRNA with the formation of a termination hairpin that stops the on-going transcription by the RNA polymerase, or (iii) attenuation based on the formation of terminator/anti-

terminator loops, which couple or uncouple the transcription by the RNA polymerase with the translation of the mRNA. Such post-transcriptional regulations are important regulatory mechanisms that are generally overlooked and underestimated, most likely because they cannot be easily investigated and estimated by transcriptomic analysis on its own (Vogel & Marcotte, 2012).

Recently, it was shown that the expression level of *agn43* can be controlled by antitermination of transcription and translation initiation in the leader mRNA (Wallecha *et al.*, 2014). Among EPS determinants, PNAG production is regulated by the RNA-binding protein CsrA (carbon storage regulatory protein A) post-transcriptionally (Boles & Horswill, 2011, Wang *et al.*, 2017), where CsrA binds cooperatively to the *pgaA* mRNA and competes for recognition with the 30S ribosomal subunit. By binding to sites located in the mRNA leader, CsrA can further destabilise the *pgaA* transcript. The transcription of *yeeJ* is increased in absence of the mRNA regulator PNPase, an exoribonuclease polynucleotide phosphorylase component of the degradosome (Martinez-Gil *et al.*, 2017).

Pili produced by the *pap* operon appears to be regulated post-transcriptionally as a result of differential mRNA stability (Baga *et al.*, 1988). The study demonstrated that the *papBA* transcript is processed and the resulting mRNA encoding the major pilin subunit accumulated. The difference in abundance of the two mRNA species could be readily explained by differences in their half-life. In *E. coli*, RNA degradation occurs *via* the degradosome thanks to the combination of endoribonuclease and exoribonuclease activities (Burger *et al.*, 2011, Bandyra *et al.*, 2013).

2.4. Regulation at the translational level

While attenuation collaterally affects the translation, three main mechanisms are directly involved in the regulation of translation, (i) anti-sense RNAs (including the small RNAs), which hybridise with mRNA and thus block the binding of the ribosome, (ii) riboregulation, where a ligand changes the mRNA folding, which consequently prevents the binding of the ribosome, and (iii) translational efficiency depending on the codon usage.

In addition to CsrA, PNAG synthesis is regulated by two small RNAs, CsrB and CsrC, which actually sequester CsrA and thus activate the translation of the *pgaABCD* transcript (Liu *et al.*, 1997, Weilbacher *et al.*, 2003). For colanic acid production, the low level of expression from the *rcsA* promoter by H-NS transcriptional silencing is alleviated by the DsrA small RNA (Sledjeski & Gottesman, 1995).

In *E. coli*, the OmpA protein is expressed to very high levels, is growth rate dependent and is a paradigm for riboregulation (Lugtenberg *et al.*, 1976, Koebnik *et al.*, 2000). Actually, the *ompA* mRNA half-life increases proportionally with the bacterial growth rate (Nilsson *et al.*, 1984). While a specific region of the transcript is targeted by the RNaseE (endoribonuclease E), binding of the ribosome induces conformational changes that mitigate the mRNA degradation (Emory & Belasco, 1990, Emory *et al.*, 1992, Hansen *et al.*, 1994). As an antagonist, Hfq can bind the transcript to decrease its stability, thus inducing RNA decay (Nilsson *et al.*, 1984, Vytvytska *et al.*, 2000). Hfq facilitates the binding of a small RNA called MicA in the vicinity of the ribosome-binding site, thus preventing ribosomal recruitment (Udekwu *et al.*, 2005).

2.5. Regulation at the post-translational level

Regulations at the post-translational level comprises the most diverse range of molecular mechanisms and is hierarchically the most important (Figure 3). In metabolic pathways, regulation at the post-translational levels is a key mechanism, particularle in relation to the modulation of the enzymatic activity, which can be influenced by physical parameters (pH, temperature, ionic force, redox, etc…), inducers and inhibitors (irreversible or reversible: competitive, non-competitive, uncompetitive or mixed inhibition) (Guedon *et al.*, 2000, Desvaux & Petitdemange, 2002, Desvaux, 2004); retro-inhibition and pro-activation can also occur and may also involve allosteric enzymes. Protein activity can be further altered by numerous post-translational modifications, namely (i) proteolytic cleavage, and (ii) chemical modifications such as disulphide bonds, phosphorylation, acetylation, methylation, adenylation or uridylation. Post-translational regulation also includes the protein folding, association/dissociation of homo- and heteromers, the degradation of proteins following the N-terminal rule by the ClpAP proteolytic complex, which can all influence the protein half-life, as well as the protein translocation to a final subcellular location. Indeed, the maturation of a protein can also occur at translocational and post-translocational levels.

As an example of post-translational regulation, the decreased production of colanic acid at 37°C results from the degradation of the RcsA transcriptional activator by the Lon protease (Ebel & Trempy, 1999). This post-translational regulation alleviates the *wca* transcription and explain the low amount of RcsA in cell (Sailer *et al.*, 2003). As a two-component system, the RcsA regulator is activated by the transfer of a phosphate group from the RcsC sensor, which is *per se* another post-translational regulation level (Desai & Kenney, 2017). For cellulose biosynthesis, the catalytic

activity of the BcsA-B complex using UDP-glucose as a substrate is allosterically controlled by cyclic-di-GMP (c-di-GMP) on the PilZ domain of the cellulose synthetase BcsA (Omadjela *et al.*, 2013). Actually, the PilZ domain was the first effector identified that is activated upon binding of c-di-GMP (Ryan *et al.*, 2012). Furthermore, the diguanylate cyclase AdrA exhibiting a GGDEF domain regulates c-di-GMP production (Romling *et al.*, 2000, Zorraquino *et al.*, 2013). C-di-GMP is a ubiquitous second messenger produced by the diguanylate cyclase exhibiting GGDEF domain, which is antagonistically degraded by the phosphodiesterases exhibiting EAL domain (Romling & Amikam, 2006). This molecule controls the motility and virulence of planktonic cells, as well as cell adhesion and persistence of multicellular communities (Jenal & Malone, 2006, Romling & Amikam, 2006, Beloin *et al.*, 2008).

As an autotransporter, Ag43 exhibits a signal peptide, which drives the preprotein to the Sec export system for translocation across the CM before being cleaved off after translocation into the periplasm. In the periplasm, several chaperones participate in the folding prior to the translocation across the OM through a cooperative mechanism involving the translocation assembly (TAM) and β-barrel assemby (BAM) machineries (Selkrig *et al.*, 2014). Additionally, the passenger of Ag43 is glycosylated, which stabilises its conformation (Sherlock *et al.*, 2006). These different post-translational, translocational and post-translocational levels all contribute to the regulation of the expression of this surface protein. While glycosylation is not that important for the functions of Ag43 (Reidl *et al.*, 2009), in TibA it is necessary for autoaggregation, adhesion to epithelial cells and biofilm formation (Cote *et al.*, 2013).

Conclusion and perspectives

Reviewing the different cell-surface molecular determinants that can participate in the surface colonisation process in DEC, from bacterial adhesion to biofilm formation, the wealth of SCFs at play is clearly highlighted. While some of these molecular determinants still remain to be fully characterised, their interplay in surface colonisation must also be carefully considered and kept in mind. The flagella, as force-generating cell-surface organelles, have been demonstrated to be important for biofilm formation (Hobley *et al.*, 2015), but expression of strong adherence factors could replace motility in the early stages of biofilm formation (Pratt & Kolter, 1998, Donlan, 2002). Although flagella expression is repressed during the switch from the planktonic to sessile lifestyle to reduce the motility capacity of the bacteria, these surface organelles have a structural and architectural role in the EPM (Hung *et al.*, 2013, Serra *et al.*, 2013). While the expression of flagellar genes are repressed, genes involved in the biosynthesis of the EPM components are generally activated during the biofilm maturation step (Guttenplan & Kearns, 2013). In *E. coli* K12, capsule polysaccharide and T1P appear to block the autoaggregation mediated by Ag43 by physically shielding intercellular Ag43-Ag43 interaction (Hasman *et al.*, 1999, Schembri *et al.*, 2004), whilst, in turn, the autoaggregation overrides bacterial motility (Ulett *et al.*, 2006). In some ExPEC, T1P expression appears to be further modulated and influenced by OmpA or OmpX, together with an increase of exopolysaccharide production, as well as a decrease in bacterial motility (Otto & Hermansson, 2004, Teng *et al.*, 2006). In NMEC, OmpA would act together with Hek in the invasion of epithelial cells (Smith *et al.*, 2007, Fagan *et al.*, 2008). All-in-all, this suggests the OMPs' composition of the OM may act as a signal in physiological adaptation of bacteria for surface adhesion and colonisation; this research direction is one of the next frontiers to be explored in DEC.

As a general trend, the average number of pili types appears lower in commensal compared to pathogenic *E. coli* (Spurbeck *et al.*, 2011). For instance, curli or conjugative pili can compensate for motility during initial adhesion and biofilm development (Prigent-Combaret *et al.*, 2000, Ghigo, 2001, Reisner *et al.*, 2003, Beloin *et al.*, 2008). Plasmids in general can encode numerous SCFs as shown in ETEC and EAEC (Amabile-Cuevas & Chicurel, 1996, Mainil *et al.*, 1998, Ghigo, 2001, Molin & Tolker-Nielsen, 2003, Kaper *et al.*, 2004, Wuertz *et al.*, 2004, Beloin *et al.*, 2008, Ong *et al.*, 2009). While conjugative plasmids can confer initial adhesion capacity and modulate the biofilm architecture (Ghigo, 2001, Wuertz *et al.*, 2004), the genetic mobility of this extrachromosomal gene pool and its contribution to biofilm formation remain poorly investigated in DEC (Dudley *et al.*, 2006). In *Pseudomonas aeruginosa*, T4P have been primarily regarded as involved in the attachment of epithelial cells in the course of an infection but later were demonstrated to also bind to abiotic surfaces such as polyvinyl chloride, polystyrene and stainless steel (Giltner *et al.*, 2006) and it even appeared to exhibit a much higher affinity towards steel than the mucosal epithelial surface, which emphasises the relevance of examining T4P in both environmental and clinical conditions (Yu *et al.*, 2007, Burgess *et al.*, 2014). In the human and animal cutaneous pathogens *Erysipelothrix rhusiopathiae*, the RspA (rhusiopathiae surface protein A) and RspB surface proteins have been shown to specifically bind several ECM components, namely fibronectin, collagens I and IV, but also polystyrene shedding light on the ecophysiology of this microorganism through its binding ability to adhere to both biotic and abiotic surfaces (Shimoji *et al.*, 2003). These aspects have not been reported or examined as yet in DEC but are particularly relevant considering the presence of T4P and ECM-binding proteins, especially some ATs, in the various *E. coli* enteropathotypes.

The regulatory network for the production of colonisation factors is often depicted as being restricted to the transcriptional level. However, this review clearly demonstrates that the range of regulation levels is much broader and even more complex (Figure 3). As a general trend, it is important to stress and keep in mind that the primary functional and regulation level is post-translational and not transcriptional, as is sometimes assumed. Whenever DNA replication, RNA polymerisation or protein synthesis occur, enzymes are essential and required for these physiological processes at pre-transcriptional, transcriptional and translational regulation levels, respectively; any abrupt changes in the environmental conditions, such as some physicochemical parameters (e.g. pH, temperature, redox potential), will have a first and direct effect on the enzyme activity before the cell can even change its transcription profile. For the SCFs, the interplay taking place at the other regulation levels is extremely complex and their hierarchy is extremely difficult to establish at a global scale. As well as this, some regulatory mechanisms in the expression of SCFs in DEC have not been fully investigated, such as attenuation, riboswitches or translational efficiency, but their involvement cannot be excluded. As molecular cell-surface determinants, the SCFs in DEC need to be translocated across a LPS-diderm bacterial cell envelope to be functional and active, which involves further translocational and post-translocational regulation levels that should not be overlooked in a regulatory network. To this end, our view of the regulatory network for the production of SCFs in *E. coli* remains incomplete and there is far from an integrated view of all regulation mechanisms. In addition, findings from investigations using domesticated laboratory strains of *E. coli* must be interpreted with caution and reinvestigation in DEC genetic backgrounds would be wise (Hobman *et al.*, 2007). This will undoubtedly lead to new discoveries in

1521 the field in the years to come and contribute to our understanding of DEC colonisation
1522 mechanisms.

1523 In DEC, SCFs have often been examined for their contribution to bacterial
1524 virulence and thus investigated in conditions related to human infection (Nataro &
1525 Kaper, 1998, Kaper *et al.*, 2004, Rossi *et al.*, 2018). In addition to humans, the GIT of
1526 a wide range of animals also harbours *E. coli* strains, both commensal and pathogenic
1527 (Escobar-Paramo *et al.*, 2006, Croxen *et al.*, 2013, Smati *et al.*, 2015, Torres, 2017).
1528 Following shedding from these animal reservoirs, *E. coli* is also found in the
1529 environment. Outside the host, the range of extraintestinal environmental conditions
1530 that can be encountered by this species is wide, ranging from soil, water to plants, as
1531 well as food matrices and food processing facilities (van Elsas *et al.*, 2011, Giaouris *et*
1532 *al.*, 2014, Jang *et al.*, 2017). As foodborne zoonotic pathogens, understanding the
1533 ecophysiology of DEC necessitates considering its lifestyle outside the human host. In
1534 fact, the role of SCFs should be placed in a context much broader than the colonisation
1535 of the GIT, as they can also play an important role in the colonisation of other
1536 environmental niches. A focus solely on the physiopathology and GIT environment
1537 may bias and limit a full understanding of the wide diversity of SCFs in *E. coli*. While
1538 the notion of virulence factors is a major contribution to the field of microbial
1539 pathogenesis (Falkow, 1988, Finlay & Falkow, 1989), a change of paradigm with the
1540 concept of coincidental by-products of commensalism (Le Gall *et al.*, 2007, Diard *et*
1541 *al.*, 2010, Leimbach *et al.*, 2013) or niche factors (Hill, 2012) is necessary to more
1542 accurately apprehend and understand the ecophysiology of pathogenic species in the
1543 food chain and in one-health approach.

1544 Taking a one-health approach considering the whole food chain, the physiology
1545 of DEC should not only be considered with respect to human infection only, but also in

1546 conditions representative of upstream, *i.e.* from the natural environments,
1547 animal/human reservoirs, agri-food environments and foodstuffs (Burgess *et al.*, 2014).
1548 Investigating the ecophysiology of the DEC with respect to the various biotopes and
1549 biocoenoses encountered in different ecosystems from natural environments, animal
1550 reservoirs, food matrices, food-processing environments, to human ingestion should
1551 shed new light on the relevance and contribution of the SCFs for this species and inform
1552 the design of strategic, targeted interventions to improve public health.

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Authors contribution statement

VA and MD wrote the first overall draft of the manuscript and draw the original pictures; RM, SL, MP, CMB, and FCD wrote sections of the manuscript. MD

contributed to conceptualise the overarching aims and had management as well as coordination responsibility for the execution of the work. MD, MP, CMB and FCD contributed to the acquisition of the financial supports and resources leading to this publication. All authors contributed to the critical revision of the manuscript, read and approved the submitted version.

Conflict of interest statement

MP is permanent employee of GSK. FCD is permanent employee of Lallemand. These authors declare that their respective company provided support in the form of salaries. All other authors declare no competing interests.

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Figure legends

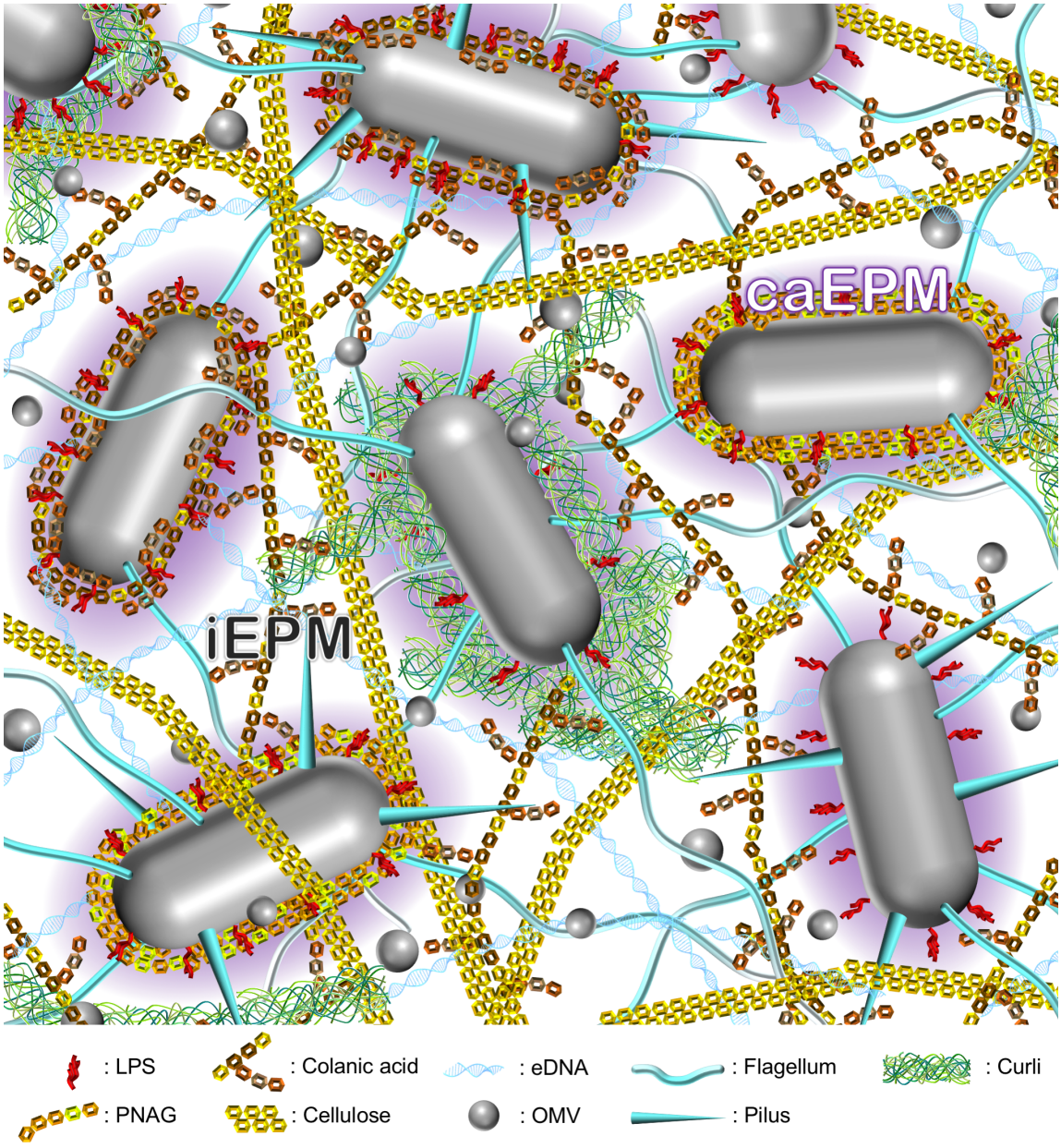
Figure 1: **Schematic representation of the exopolymeric matrix (EPM) in *E. coli* biofilm**. By analogy with the extracellular matrix (ECM) in mammalian tissue, the EPM in bacterial biofilm can be further discriminated between (i) the EPM closely associated with the bacterial cells, i.e. the cell-associated EPM (caEPM) (purple shade background), and (ii) the interstitial EPM (iEPM) (white background). Molecular determinants of the caEPM are attached, anchored or linked to the bacterial cell surface. Besides cell-surface proteinaceous determinants including monomeric proteins (not depicted in the picture) and supramolecular protein structures, such the flagella and pili, molecular components of caEPM further comprise extracellular polysaccharides (EPS), namely some lipopolysaccharides (LPS) as well as poly-β-1,6-N-acetyl-D-glucosamine (PNAG) and colanic acid, which both form a capsule. Together with colanic acid that can be released from the bacterial cell surface, cellulose can compose the EPS part of the iEPM. Besides extracellular DNA (eDNA), some exoproteins (not depicted in the picture) and outer membrane vesicles (OMV) may also constitute the iEPM in *E. coli* biofilm.

Figure 2: **Schematic representation of the cell-surface proteinaceous determinants acting as CFs in DEC**. Monomeric proteins are depicted in shades of red, whereas multimeric protein complexes are depicted in shades of blue. Whenever possible, molecular structures were obtained from the protein databank (PDB) (Berman *et al.*, 2002, Rose *et al.*, 2017) or the electron microscopy databank (EMBD) (Lawson, 2010). Regarding ATs, no structure for ATAs is currently available but Ag43 (PDB: 4KH3) is provided as a representative of a SAAT and EspP (PDB: 3SLI, 3SZE) as a representative of SPATE. Intimin (PDB: 3NCW, 4E1S) is given as a representative of

an IAT. Proteins secreted across the OM by the T5SS are first exported *via* the Sec translocase (SecYEG-DF/SecA) (PDB: 2AKH, 3AQO, 5XAM) across the inner membrane (IM). Dispersin (PDB 2JVU) is secreted *via* T1SS (PDB: 5066). Besides ATs, all OMPs including the Hra, OmpA (PDB: 2GE4) and Iha are first exported *via* Sec before being integrated into the OM *via* the β-barrel assembly machinery (Bam) complex (BamABCDE) (PDB: 5LJO). The surface-associated lipoprotein of *E. coli* (SslE) is secreted by a T2aSS (EMDB: 1763, PDB: 3CIO, 3OSS, 4KSR, 2W7V, 2BH1) after Sec export. Like the moonlighting proteins represented here by GAPDH (PDB: 5ZA0), the secretion mechanisms of Efa-1 remain unknown. EibD (PDB: 2XQH) is provided as a representative of TAAs. The injectisome is secreted and assembled by the T3aSS (EMDB: 1875). The flagellum (EMDB: 1132, 1873; PDB: 1IO1) is secreted and assembled by the T3bSS (EMDB: 1887). The T4P (EMDB: 0070) is secreted and assembled by the T2bSS. The conjugative pilus (CP) (PDB: 5LEG) is secreted and assembled by the T4bSS (EMDB: 2567). The T1P (EMDB: 3222), CS31A, AAF (PDB: IUT2, 2XQ), CFA (EMDB: 1952), F9 pilus, ELF, LPF (PDB: 5AFO), ECP (PDB: 3QS3) and SFP are all secreted and assembled by T7SS (PDB: 4J3O) after Sec export. The curli are secreted and assembled by the T8SS (EMDB: 2750). Hcp form a tube, which is displayed extracellularly upon triggering of the T6SS (EMDB: 2524; PDB: 4HKH, 3RX9, 4JIV).

Figure 3: **Regulation levels and control mechanisms for the expression of genes encoding colonisation factors in DEC.** Respective to biochemical process, the sequential steps and events for gene/protein expression flow from pre-transcriptional, transcriptional, post-transcriptional, translational to post-translational regulation levels (as depicted by blue arrows). Thus, at least five regulation levels can be considered in bacteria and at each level, different control mechanisms can be at play. Besides, for a

same protein encoded gene different regulation levels and regulatory mechanisms can intervene, *e.g.* the expression of Ag43 is regulated at pre-transcriptional level by DNA methylation, at transcriptional level by OxyR, at post-transcriptional level by antitermination of transcription and translation initiation in the leader mRNA, and also at post-translational levels with its autoaggregative activity modulated by pH, its native folding requiring chaperones and final subcellular localisation by translocation across the OM. Besides rRNA, tRNA and sRNA, biological functions and activities are essentially represented by proteins and the hierarchy of regulations levels and control mechanisms (as depicted by shades of red) is opposite to the gene/protein expression flow; e.g. whatever the pre-transcriptional (with DNA replication), transcriptional (with mRNA synthesis), post-transcriptional (with the modulation of transcripts) or translational (with the protein synthesis) levels, they are all strictly depend on enzyme activites which can be regulated at post-translational levels in the first place with direct and immediate effect due to modulation of their catalytic activity by temperature or pH for instance.



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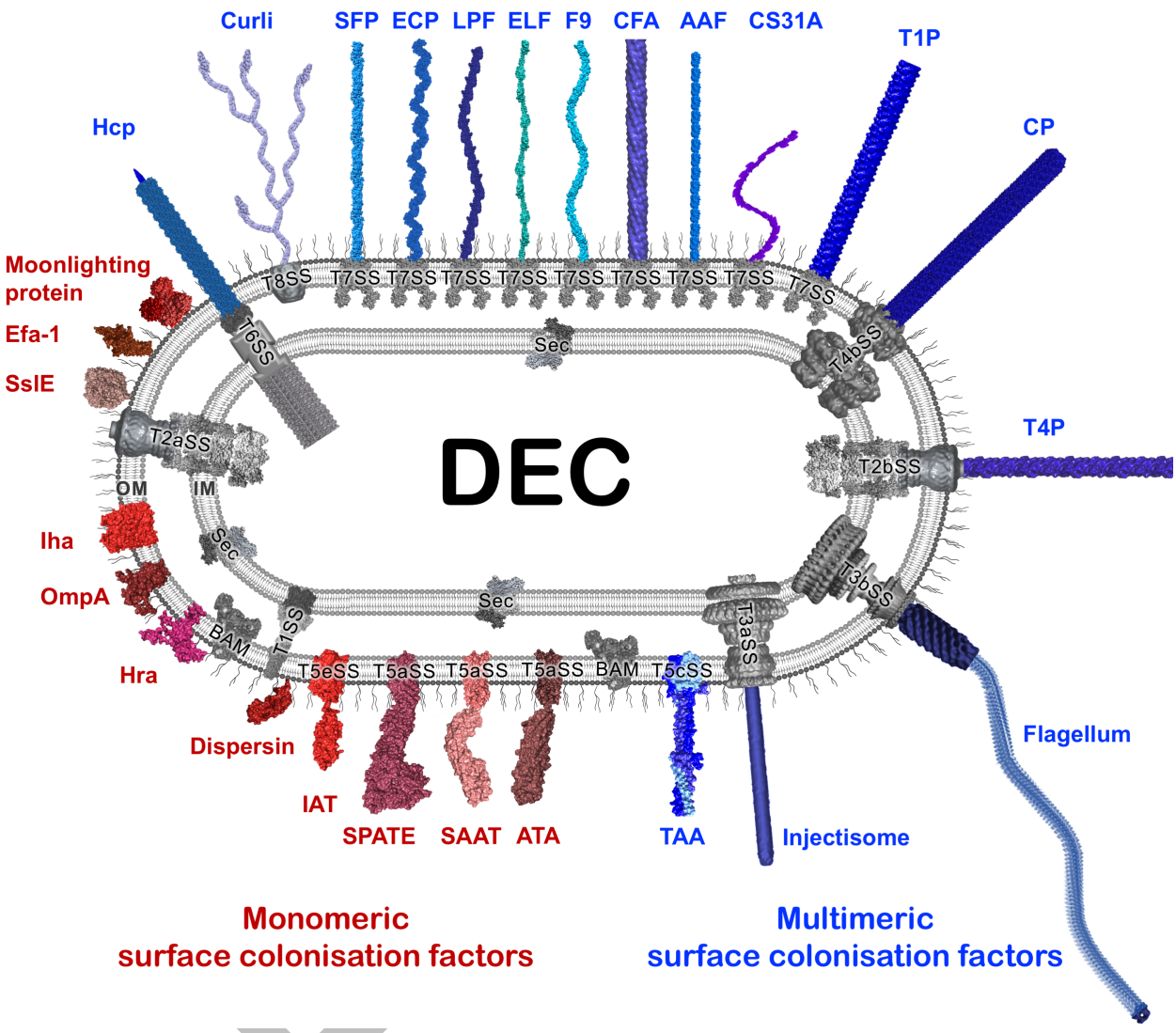
3433 Figure 1



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