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Genetic and Phenotypic Analysis of *Escherichia coli* K-12 Adhesion to Inanimate Surface

Thesis

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Chapter I

General Introduction and Outline of the Study

GENERAL INTRODUCTION

Adhesion to Surfaces: A Bacterial Point of View

The natural tendency of bacteria to adhere and colonize a variety of surfaces is regarded as a fundamental aspect of bacterial pathogenesis and ecology (14). For many human, animal and plant pathogens, adhesion to the host surface is a necessary step for successful colonization and therefore, directly or indirectly, for the elaboration of diseases (26). Numerous bacterial proteins, called adhesins, which recognize specific receptors on host cells or the extracellular matrix, have been characterized (42, 36, 48). In addition, other bacterial surface structures such as flagella, lipopolysaccharides (LPS) or exopolysaccharides (EPS) were found, in some cases, to participate in host colonization (60, 64, 25).

In addition to specific host-bacteria interactions, the ability to adhere to abiotic surfaces is regarded as an important characteristic of microorganisms (14). Formation of bacterial films on surfaces can be desirable, as for wastewater treatment or microbial immobilization in bioreactors (94, 16), but can often lead to serious economic and health problems. Colonization of medical devices by opportunistic pathogens has been found to be a critical step in the appearance of various infections (11, 53, 14). Accumulation of microorganisms on tooth surfaces causes common human diseases such as caries and inflammatory periodontal disease (101). In the diary industry, biofouling of surfaces causes sanitation problems and a decrease in biocidal treatment effectiveness (108). Adherence of bacteria to surfaces in contact with flowing systems such as ships, platforms, pipelines, and heat exchangers, often results in serious fouling and corrosion problems (14).

Adhesion to inanimate surfaces is a complex phenomenon in which microorganisms can interact with various physico-chemically-different substrata under a plethora of environmental conditions (41). This process usually takes place in two principal steps: the initial attachment and the subsequent formation of the biofilm matrix. The initial binding that requires cell-surface interactions often appeared to be influenced by physico-chemical factors such as cell surface charge, hydrophobicity and the nature of the substratum (70, 91). Cell surface structures such as pili, flagella, non-pilus proteins, LPS and EPS were identified as active participants in the initial adhesion reactions, either by directly performing attachment or

by simply increasing the chance of contact with the substratum. The bacterial structures concerned can act together, in an antagonistic manner, or simply be non-required for attachment to a given surface. Biofilm matrix formation, which corresponds to a more stable establishment of the bacteria onto a surface, requires the formation of cell-to-cell bridges. Some pilus and non-pilus adhesins were found to actively participate in intercellular adhesion (coaggregation) within cells of single or different bacterial species, by recognizing specific receptors on the host cell (99). EPS also often participate in biofilm matrix formation and maintenance by holding the bacteria in the biofilm structure and permitting an efficient contact with the liquid phase (15).

Herein are presented diverse bacterial structures such as pili, flagella, LPS, EPS and other non-pilus adhesins involved in the processes of primary adhesion and biofilm matrix formation. The role(s) of these structures in adhesion to inanimate surfaces is described and compared, when necessary, with their purpose(s) in adhesion to living surfaces.

Bacterial adhesins

Bacterial adhesins are characterized proteinaceous structures that perform attachment to host specific receptors. The adhesins can be found as monomers, as simple oligomers or on a fibril structure.

Pilus adhesins:

Bacterial fimbriae or pili (from the Latin words "threads" and "hairs" respectively) are long proteinaceous hairlike surface organelles (about 100 to 1000 fimbriae per cell) ranging in diameter from 2 nm to 10 nm found in many bacterial species (23). The principal characteristic of these organelles is to posses specific adhesive properties. The fibrils are often composed of major structural proteins that present a tip-associated adhesive protein (adhesin) to the host cells, e.g. P-fimbriae. In some cases, the adhesin was located along and at the tip of the fimbrial rod (e.g., Type 1 fimbriae) or was the major structural protein itself (e.g. K88, K99 and CFA/I fimbriae). Fimbriae adhesins usually bind to specific cellular or extracellular carbohydrates and to proteinaceous targets (37, 90). Interaction with the host is a very selective characteristic that may reflect the restriction of some organisms for a specific environment (37). Adherence to host cells provided by fimbriae is often required for successful colonization and therefore for infection by various microbial pathogens. The fimbrial diversity, the molecular biology and the

interactions of such organelles with the host cell, extracellular matrix or mucus have been extensively studied and will not be discussed here (for recent books and reviews see 37, 42).

The role of bacterial fimbriae in adhesion to abiotic surfaces has been only rarely reported. Rosenberg *et al.* (78) first reported the involvement of *Acinetobacter calcoaceticus* fimbriae in adhesion to hydrophobic surfaces such as polystyrene and hydrocarbons. The strain possesses one type of "thick" fimbriae (5 nm) and one type of "thin" fimbriae (3 nm). The thick fimbriae associated with twitching motility did not confer adhesion, whereas the thin fimbriae constituted the crucial adherent factor of the strain (78). In contrast, fimbriated *Serratia marcescens* strains exhibited a lower degree of hydrophobicity and adherence to an air-water interface than the non-fimbriated one (32). The P-pilus, that binds specifically to a Galα(1-4)Gal disaccharide moeity via the adhesin, PapG, localized at the tip of the organelle, is commonly associated with epithelial cells and appears to be involved in various infections (37). The presence of P pili is not associated with the adhesive behavior of various isolates of *Escherichia coli* from urinary tract infections to polystyrene and urinary catheter material (silicone latex) (29, 76). The mannose-sensitive type 1 fimbriae (haemagglutination patterns inactivated by the presence of D-mannose) are made of about 1000 components of a major subunit protein FimA and of three minor ones, FimF, FimG and FimH which comprise about 1% to 2 % of the fimbrial proteins (43). The adhesin FimH, associated with the two other minor subunits, is integrated at the tip and along the fimbrial rod (46). FimH was found to specifically bind mannose-oligosaccharides via carbohydrate interactions, and fibronectin via both carbohydrate and protein interactions (90). The first report concerning adhesion to an inanimate substratum involving type 1 fimbriae by Brinton (9) showed that purified type 1 fimbriae from *E. coli* K-12 were able to agglutinate polystyrene latex spheres. Early adhesion to polystyrene of various urinary isolates of *E. coli* was maximal for strains possessing mannose-sensitive fimbriae (29). In addition, adhesion was strongly inhibited by D-mannose. Genevaux *et al.* (*Chapter III*) isolated various Tn*10* insertion mutants of *E. coli* K-12 that exhibited a strongly altered attachment to polystyrene independent of the time, of the cell age, and of the cell number. These adhesion mutants were mutated in the *fim* operon encoding for type 1 fimbriae biosynthesis and did not produce fimbriae. In addition, adhesion of the fimbriated wild type strain was strongly inhibited by the addition of D-mannose (the receptor of the FimH adhesin of type 1 fimbriae) into the medium (29, chapter III). Studying *Salmonella typhimurium* adhesion to mineral particles such as albite, biotite, feldspar, magnetite and quartz,Stenström *et al.* (91) showed that the presence of type 1 fimbriae conferred a higher degree of adherence. Similar observations were made for the type 1 fimbriae of *Klebsiella* strains. Jansen *et al.* (40),

showed that adhesion to polyetherurethanes (synthetic polymers frequently used in medical devices) of *Klebsiella* strains possessing type 1 fimbriae was much stronger than that of the non-fimbriated variants. In this case also, the addition of mannose into the medium inhibited. Thus, the results obtained with type 1 fimbriae of *S. typhimurium*, *Klebsiella* and *E. coli* indicate that the role these organelles in adhesion to inanimate surfaces is of importance (91, 40, *Chapter III*). Furthermore, the mannose-sensitive adhesion observed shows that the FimH adhesin itself appears to be an important determinant in synthetic polymer colonization. More knowledge about specific inhibitors of fimbrial adhesion to synthetic polymers, such as mannose in the case of type 1 fimbriae, may help to counteract undesirable adhesion and further biofilm development on medical, industrial or environmental surfaces.

Aggregative Pilus:

An other class of surface organelles in *E. coli* and *Salmonella enteritidis*, called curli or aggregative pili, has been identified (13, 65). In contrast with bacterial fimbriae that are assembled from the base, aggregative pili are formed outside of the cell by precipitation of secreted soluble proteins into thin fibers (37). These structures are often produced below 37°C in low osmolarity media during stationary phase (3). The biological role of bacterial curli is not yet known. They appear to bind fibronectin, laminin and plasminogen and to have autoaggregative tendencies due to their hydrophobic nature (37). Such structures were recently found to adhere to polystyrene and glass surfaces and to build up strong biofilms (97).

Non-pilus adhesins:

Many bacterial pathogen adhesins are not assembled into polymeric pilus rods as described above, and are called non-pilus adhesins. These non-pilus adhesins are usually presented on the outer membrane of the bacterial cell as monomers or simple oligomers, and are named AFA (afimbrial adhesin) or NFA (non-fimbrial adhesin). They can form, in some cases, an amorphous capsule-like material composed of large multi-unit aggregate proteins. As for fimbrial adhesins, the recognition of host receptors appears to be fine-tuned. These adhesins are found to recognize mostly determinants of the M/N or of the Dr (a) blood groups, and to exhibit a mannose-resistant (MR) specificity (for reviews see 26, 36, 83). The involvement of non-pilus adhesins in adhesion to inanimate surfaces has been rarely studied. Hoght *et al.* (33) observed that attachment to biomaterial surfaces, as well as the hydrophobicity of *Staphylococcus epidermidis*, were reduced in the presence of proteases. Heilmann *et al.* (30) found a 60 kDa surface protein which mediates primary attachment to relatively hydrophobic

surface such as polystyrene, but not to a more hydrophilic glass surface. Timmerman *et al.* (95) identified two other proteins of *S. epidermidis*, called SSP-1 and SSP-2 (280 and 250 kDa respectively), localized on the bacterial surface and on "fimbriae-like" surface projections, that mediate attachment to polystyrene surface. Further study showed that SSP-1, rather than SSP-2, was the major adhesin, and that proteolytic cleavage of SSP-1 produced an SSP-2-like protein and therefore a loss of adhesion. The switch in adherence from an active SSP-1 to an inactive SSP-2 may reflect the ability to escape from an unfavorable environment (96). Although they are rarely mentioned as direct participants in the process of primary adhesion to inanimate surfaces, many of the non-pilus adhesins (as well as pilus adhesins) were found to be involved in cell-to-cell recognition (coaggregation) and therefore to promote formation of intra- and inter-species biofilms (38, 99).

Flagella and motility

Bacterial flagella provide movement and orientation to the cell in response to environmental stimuli. The flagella structure, the number, and the response to external stimuli can vary considerably among species (see 60 for a review). Flagella and motility have often been associated with colonization of both biological and non-biological surfaces, but the precise contribution of these organelles in the process is not clear yet.

Motility often influences infection by virulent species. In *Aeromonas hydrophila*, a primary pathogen of a variety of aquatic and terrestrial animals including humans, motility was an important factor for adherence to fish cell lines, whereas a direct role of flagella as an adhesin remains hypothetical (56). Colonization of the *Euprymna scolopes* light organ by *Vibrio fischeri* strongly required motility, and the presence of flagella alone was not sufficient for colonization. This indicated that bacteria were not simply swept by the host ciliary movement, but were active participants in the colonization process (27). In *Agrobacterium tumefaciens*, flagella were required for motility to facilitate reaching the root surface, and possibly aid in orientating the cells at various sites for infection. Bald flagella insertion mutants harboring the tumor-inducing Ti plasmid were still virulent, but consistently less than the parent strain (10). The Gram-negative spiral-shaped *Helicobacter pylori*, an important etiologic agent of gastroduodenal disease in humans, are highly motile. This property is considered to be an important factor in the colonization ability of the bacteria (24).

Some virulence factors have exploited the flagella export system and/or share common regulatory features with the flagella system. Thus, a direct role of flagella and motility in adhesion has been difficult to address. Mutations affecting flagella assembly proteins that are conserved in plant and animal pathogens reduced virulence of *Erwinia carotovora in planta* (63). The authors propose that the flagellar specific export pathway has been exploited as transmembrane targeting route for virulence factors (63). Yao *et al.* (105), showed that paralyzed flagella of *Campylobacter jejuni* were able to mediate adherence but not to perform invasion. However, motility, rather than the flagellin FlaA, was necessary for invasion of epithelial cells. Invasion may involve adhesins that are part of a motility-dependent invasion process (105). In *Bordetella bronchiseptica*, expression of different virulence factors is coordinately regulated by the *bvg* locus (*b*ordetella *v*irulence *g*enes) (1). Flagella have been found to be regulated by this locus and to perform adhesion to human epithelial cells, but the exact role of the flagella in *B. bronchiseptica* virulence has yet to be described (82). The mechanisms by which *Pseudomonas aeruginosa* colonizes human lungs are not well understood, but appear to be mediated by non-pilus adhesins that bind to mucin. However, mutations in genes involved in the flagellar export apparatus or in basal body structural components resulted in a loss of adhesion as well as motility (4). Arora *et al.* (5) found two transcriptional regulators, FleQ and FleR, in *P. aeruginosa* which regulated both expression of mucin adhesin(s) and the expression of flagellar genes. In *Proteus mirabilis*, flagella contributed significantly, but not alone, to the ability to colonize the urinary tract (59). A*flhA* mutant of *P. mirabilis* (homologous to the *E. coli* FlhA protein required for flagella assembly) was non-motile and considerably affected in the expression of the virulence *hpmA* gene, encoding haemolysin toxin. These results suggested that flagella and haemolysin, and probably other virulence factor's expression, are closely coupled (28). In *S. typhimurium*, non-flagellate mutants were found to be less virulent and less invasive (49). Schmitt *et al.* (85) showed that virulence, but not motility, of *S. typhimurium* was attenuated by a single mutation in the flagella gene *flgM*. This conserved gene in *Salmonella* species is involved in negative regulation of flagellin synthesis by inhibiting the flagellar-specific sigma factor *fliA* gene when mutations in flagellar basal body, hook, or switch genes were present. In a motile *flgM* mutant the wild-type virulence was restored by a secondary mutation in the positive flagellum regulator *fliA*, whereas the *fliA* mutation alone had no effect on virulence, but did confer a non-motile phenotype. These results suggest that the virulence-associated target gene(s) and flagellar synthesis in *S. typhimurium* may share regulatory features (84, 85).

Flagella and motility have often been associated with adhesion to non-biological surfaces. The marine bacteria *Vibrio alginolyticus* and *Vibrio parahaemolyticus* possess two flagella systems, i.e. a polar flagellum and lateral flagella. Cells in liquid phase swim by using the polar flagellum. This flagellum is constitutively synthesized and is sheathed with a membrane similar in composition to the outer membrane. Recently, Kogure *et al.* (44) showed that the polar flagellum conferred faster attachment, but that the organelle does not serve as an appendage for attachment. The authors concluded that attachment to glass surface was dependent on the swimming speed. The lateral flagella are synthesized only when cells are on surfaces or in a high viscosity environment, and propel the bacteria over the surface (swarming). Once the cells are in contact with the surface, the newly synthesized lateral flagella may influence the stronger adhesion of the marine *Vibrio* (6, 89). However, a direct role of lateral flagella as adhesins has yet to be described (44). Adhesion of *Pseudomonas fluorescens* to glass surface was about four times more rapid for motile strains than for non-motile ones (45). Furthermore, the final cell densities on surface were higher for the motile strain (45). The authors concluded that motility conveyed a selective advantage during surface colonization, but in that case motility alone was considered, and no other adhesion factors that could have been affected by the loss of motility, as discussed above were taken into account. Tn*5* insertion mutants of *P. fluorescens* defective in adhesion to soil and seeds were also affected in flagella biosynthesis (19, 20). These mutants were defective in the initial stages of attachment and were affected in motility capabilities, but not for flagella biosynthesis. In *E. coli*, the structure of the flagella and the response to environmental stimuli are well-understood (see 51 and 93 for review). However, the interactions of these organelles with surfaces are as yet not well defined. Adhesion to polystyrene surface of urinary isolates of *E. coli* showed that non-flagellate and de-flagellate strains produced only a weak adhesion to polystyrene when compared to motile ones (29). The authors suggest that flagella may play an active role in the attachment process, more than increasing the chance of cell-surface contact. A recent study of various adherent Tn*10* insertion mutants of *E. coli* K-12 showed that motility was involved in the initial step of adhesion to polystyrene, probably increasing the chance of contact with the surface, whereas a direct involvement of flagella as an adhesin was not observed (*Chapter III*). The weak final adhesion of flagella mutants was not due to the absence of flagella, but to the effect of the flagella mutations on type 1 fimbriae biosynthesis (*Chapter III*). Thus, in *E. coli* K-12, as in some pathogens in which virulence factors share regulatory features with the flagella system, the loss of flagella and/or motility, directly and indirectly controls the adhesion capacity of the bacterial cell.

The production of flagella appears to be an advantage for adhering bacteria. Motility provided by functional flagella often appeared to confer an advantage during colonization. A direct role of adherence for bacterial flagella has been, in some cases, postulated but has yet to be proved. In addition, the shared common regulatory features observed between the flagella system and other surface adhesins considerably influence the adhesion process. This last point indicates that the study of the effect of flagella and motility on adhesion or invasion requires more knowledge about other cell factors (pilus or non-pilus adesins) that can be co-regulated with the flagella system.

Bacterial polysaccharides

Lipopolysaccharides:

Lipopolysaccharides (LPS: endotoxins) are immunogenic glycolipid cell wall constituents of most Gram-negative bacteria and are necessary for functioning of this membrane. LPS is the major surface antigens and has immunostimulating and immunomodulating compounds (see 34 and 62 for review). These glycolipid structures are composed of three distinct structural regions, from the cell outer membrane to the external medium: the lipid A, the core, and the O-antigen. The lipid A is composed of fatty acid and phosphate substituents bound to glucosamine dimers, and represents the hydrophobic anchor for the LPS into the outer membrane of the bacteria (74), as well as the endotoxin principle of the LPS involved in some pathophysiological reactions (34). The LPS core region is a branched non-repeating oligosaccharide required for membrane stability and functionality of some surface proteins and organelles (69, 58, 98, *Chapter IV*), and for resistance to certain antibiotics and detergents (67, 74). The O-antigen consists of an immunogenic polysaccharide chain of repeating units of 2 to 8 sugar monomers, which vary considerably between species (34, 74). More details about the biochemistry and genetics of LPS are described elsewhere (74, 86).

Apart from their antigenic role, LPS, and principally the O-chain, appeared to play a role in adherence but a precise contribution of LPS in host cell colonization has been difficult to establish. However, correlation between the lack of O-side chain and an increased adherence has been found. *In vitro* studies of *S. typhimurium* showed that an *rfaJ* mutant lacking the O-antigen part of the LPS could adhere to epithelial cell preparations at a rate three to five times higher than the wild-type (64). These differences were not caused by motility alteration (55). However, the wild-type LPS conferred a better penetration of the intestinal mucosal layer in *in*

vivo conditions (47). Pierson (68) isolated Tn*5B50* mutants of *Yersinia enterocolitica* that lacked the O-side chains of the LPS and showed that the truncated LPS mutants had enhanced adherence and entry into mammalian cells. The author suggested that the truncated LPS may have over-exposed the cell entry factors (68). However, the O-side chain of *E. coli* O157:H7, a strain often associated with a spectrum of illnesses in humans, was not involved in attaching and effacing adherence to epithelial cells *in vitro* (12).

An involvement of LPS in adhesion to abiotic surfaces has also been observed. The loss of the O-polysaccharide chain was found to significantly increase adhesion to relatively hydrophobic components. The increased adhesion to various hydrocarbons observed for a rough mutant (LPS lacking the O-chain) of *E. coli* was presumably due to an increased exposure of the inner core regions of the LPS (79). Studies of the hydrophobic interactions in *S. typhimurium* showed that cell surface hydrophobicity of various rough mutants and deep rough mutants (core truncation of the LPS) was higher than in the parent (smooth LPS). These results suggested that the lipid A moiety probably was the main hydrophobic reaction site (52). Hermansson *et al.* (32) observed that the O-side chain of *S. Typhimurium*, and less so the core, participated to the hydrophilic character of the bacteria. More recently, similar effects were observed by Williams *et al.* (100). Transposon mutagenesis, with Tn*PhoA* was used to produce *P. fluorescens* mutants with altered adhesion ability and transport through porous media (100). Adhesion mutants showed an increased attachment to hydrophobic surfaces and a decreased attachment to more hydrophilic ones. Cell surface characterization of the mutants showed a complete loss, or consistent attenuation, of the O-antigen of the LPS. In *E. coli* K-12, that contains no O-side chains but only lipid A and the polysaccharide core (rough LPS), mutations affecting the LPS core were found to decrease attachment to polystyrene (*Chapter IV*). Further analysis showed that the consequent loss of adhesion exhibited by these mutants was mainly due to the pleiotropic effect of the LPS mutations on type 1 fimbriae biosynthesis, whereas a direct effect of the LPS core alterations on adhesion was not observed.

Exopolysaccharides:

In addition to the LPS, bacterial polysaccharides can be produced by bacteria as an amorphous layer of extracellular polysaccharides (EPS) which are secreted around the cell either as capsule or as loose slime. EPS are highly hydrated and considerably heterogeneous molecules. They can form homo or heteropolymers, can be branched or unbranched, and can be substituted by organic and inorganic molecules. Many functions have been suggested for EPS. The capsule may protect the bacteria from desiccation, may confer resistance to several toxins,

phages, and specific and non-specific host immune defense. Furthermore, many phytopathogenic microorganisms produce EPS, which has been found to be essential for virulence (see 77 for review). In adhesion and biofilm formation processes, the role of EPS is of importance. Some EPS have been associated with the initial adhesion, but most of them appeared to be synthesized by attached bacteria as an intercellular adhesion factor necessary for construction of the biofilm matrix or in some cases as mechanisms to evade from an inappropriate environment (see below).

A capsule-like extracellular material of *Klebsiella pneumoniae* was found to mediate aggregative adhesion to human intestine cells and therefore to contribute to the maintenance of the bacteria inside the gastrointestinal tract (25). A non-capsulated *Haemophilus influenzae* adhered more to an epithelial cell line than the encapsulated parent (92). The type III capsular polysaccharide of group B streptococci, which allowed the organism to evade major host defense mechanisms, was not involved in respiratory epithelial cell invasion (35). Jacques *et al.* (39) showed that virulence of *Pasteurella multocida* in mice and piglets increased with the synthesis of capsule, whereas no evidences for a role of this capsule in adherence to respiratory tract cells and mucus could be observed. Study of enterotoxigenic *E. coli* adherence to intestinal epithelial cells of pigs showed that A-type encapsulated strains adhered less than the non-encapsulated ones. In this case, the presence of a capsule appeared to impede pilus-mediated adhesin (81). *S. epidermidis*, which is part of the normal skin and mucosal microflora, emerges as cause of various infections associated with the use of catheters and other medical devices. The capacity of S. *epidermidis* to form biofilms on medical devices is regarded as the major cause of these infections (11). The initial adhesion was found to be correlated with the presence of surface proteins (30, 95, 96). The second stage, consisting in biofilm matrix formation, has been found to be mediated by three intercellular adhesion genes (*icaABC*) that encode for the biosynthesis of a polysaccharide intercellular adhesin (PIA) (31, 50). In addition, the *ica* genes were recently found to undergo phase variation (107). The switch in PIA production reflects the adaptive potential of *S. epidermidis*. The opportunistic pathogen *P. aeruginosa* has been shown to cause serious infections of the respiratory tract in patients with cystic fibrosis (21, 54). The appearance of mucoid strains overproducing an alginate EPS generally coincided with these infections. The produced alginate EPS conferred advantages in the ability to adhere to tracheal cells (54, 75). The ability of this bacterium to adhere and colonize medical devices is a crucial factor in infection (18, 53). Alginate EPS has been shown in many cases to improve attachment and anchoring to solid surfaces such as darcon fiber, teflon and glass (8, 17, 18, 53). Davies *et al.* (18) showed that cells attached to a glass surface

rapidly up-expressed the alginate biosynthesis gene *algC*, but that initial cell attachment appeared to be independent of *algC* activity. Furthermore, the expression of the alginate gene was correlated with the ability of bacteria to remain on the substratum. While the influence of alginate EPS on initial attachment is difficult to establish, its role in biofilm growth and maintenance appeared to be of importance. In addition, Doig *et al.* (22) reported that some alginate purified from different *P. aeruginosa* strains could bind to buccal and tracheal epithelial cells, whereas some could not. These results may reflect the crucial role of alginate structure in the ability to perform attachment. Furthermore, *P. aeruginosa* is capable of degrading its alginate polysaccharide by synthesis of a periplasmic lyase, AlgL. Induction of the alginate lyase reduced the size of the EPS and increased detachment of the cell (7). This process may reflect a way to escape from an unsuitable environment. An alginate EPS producing *P. fluorescens* appeared to have enhanced affinity for water and reduced adhesion to relatively hydrophobic surfaces. In this case, the EPS may not promote primary attachment but may participate to the development of the biofilm matrix, which requires the formation of cell-to-cell bridges (71). The EPS of freshwater bacterial isolates were also found to enhance development of the microbial film but were not required for the initial attachment to a glass surface (2). Rosenberg *et al.* (80), showed that the EPS of *A. calcoaceticus* decrease adhesion to hydrocarbons, presumably by increasing their affinity for the water phase. The marine *Pseudomonas* sp. strain S9 was found to produce two forms of a neutral EPS, one integral form closely associated with the cell surface produced during both growth and starvation, and a loosely associated peripheral form produced only during starvation. Biosynthesis of the peripheral form during starvation was found to affect adhesion to hydrophobic inanimate surfaces and therefore to increase detachment (102, 103, 104). The authors proposed that starvation induced a specific response that assembles EPS into larger subunits detaching the cells from the surface (104). In the oral cavity, most of the bacteria exhibit properties of intergeneric coaggregation that involves many lectin-like adhesin proteins that recognize specific saccharide receptors at the cell surface of other bacterial species. In this case, bacterial polysaccharides considerably participate to dental plaque colonization (for more details see 99). The fouling bacterium *Deleya marina* produces large quantities of EPS. This EPS has been found to facilitate cell aggregation and to increase adhesion to more hydrophilic substratum (87, 88). Marine *Caulobacters* participate in biofouling phenomena by the production of holdfast containing polysaccharides that are assumed to mediate strong attachment to various surfaces (57, 66, 106). The marine bacterium *Hyphomonas*, considered as a primary colonizer of surfaces, produces a capsule-like EPS, which is associated with flocculation and formation

of thick biofilms on both hydrophilic and hydrophobic surfaces. In this case, the EPS appears to act as primary adhesin and to perform biofilm development (72, 73).

The involvement of EPS in initial adhesion and biofilm formation is significant. In many cases, it appears to be essential for the construction of the biofilm matrix, providing cell-to-cell bridges once the bacteria are attached to the substratum. Furthermore, the ability of EPS to bind ions and nutrients may contribute to biofilm growth and maintenance. Do EPS directly actes as adherent? This property may depend on the chemical structure of the EPS and in the nature of the surface. In addition, EPS can be synthesized by attached bacteria as a mean to escape from inappropriate environments, or degraded for the same proposes.

Concluding remarks

It clearly appears that bacteria can utilize different mechanisms and structures to adhere to different surfaces. Motility often confers selective advantages during colonization, whereas a direct role of flagella as adhesin has yet to be described. Furthermore, the common regulatory features shared among flagella and some adhesins such as pili or other virulence factors is of importance and must be considered. Pilus and non-pilus adhesins, mostly considered as specific bacteria-host cell interactions and coaggregation factors, also appear to play a considerable role in adhesion to inanimate surfaces. LPS were found, in some instances, to increase affinity for the water phase. Some types of EPS are direct cell-surface adhesins, but mostly confer cell-to-cell bridges. Some attached bacteria can synthesize EPS as a mean to escape from inappropriate environments. The huge amount of membrane structures, their diversity among species, and the ability to induce specific changes in response to an undesirable environment reflects the adaptive potential and the preferences for specific econiches expressed by some microorganisms. More data are needed to better understand how these structures are acting and interacting. The genetic approach, successfully used for identification of many specific adhesins in studying adhesion to living cells, may provide further insight.

OUTLINE OF THE STUDY

The very well characterized gram-negative bacterium *E. coli* K-12 was used to identify genes and membrane structures involved in colonization of inert surfaces.

- In *chapter II* a random insertion mutagenesis using a mini-Tn*10* transposon was performed in *E. coli* K-12. A rapid screening assay, based on staining of adherent cells to a polystyrene surface, was developed and used to identify non-adhesive mutants.

- *Chapter III* presents the procedure used to precisely localize the transposon in the genome of the adhesion mutants. Tn*10* insertions in the *fim* operon (encoding for the type 1 fimbriae) and in various flagella genes that affected adhesion to polystyrene are described, and the role of these structures and their interactions in the adhesion process is investigated.

- *Chapter IV* describes Tn*10* insertions located in genes involved in LPS core biosynthesis that affect *E. coli* adhesion. The pleiotropic character of these mutations is described and the role of LPS in adhesion to inanimate surfaces is discussed.

- In *chapter V*, three adhesion mutants in which the Tn*10* was located in the *dsbA* gene are described. The indirect multi-effect of the *dsbA* mutations on adhesive structures is presented.

- *Chapter VI* describes five adhesion-deficient mutants with Tn*10* insertions located in the *phnI*, *yhhT*, *hisT*, and *abc* genes, and in IS *186*.

References

1. **Akerley, B. J., D. M. Monack, S. Falkow, and J. F. Miller.** 1992. The *bvgAS* locus negatively controls motility and synthesis of flagella in *bordetella bronchiseptica*. J. Bacteriol. **174:**980-990.

2. **Allison, D. G., and I. W. Sutherland.** 1987. The role of exopolysaccharides in adhesion of freshwater bacteria. J. Gen. Microbiol. **133:**1319-1327.

3. **Arnqvist, A., A. Olsen, J. Pfieffer, and S. Normark.** 1992. The Crl protein activates cryptic genes for curli formation and fibronectin binding in *Escherichia coli*. Mol. Microbiol. **6:**2443-2453.

4. **Arora, S. K., B. W. Ritchings, E. C. Almira, S. Lory, and R. Ramphal.** 1996. Cloning and characterization of *Pseudomonas aeruginosa fliF*, necessary for flagellar assembly and bacterial adherence to mucin. Infect. Immun. **64:**2130-2136.

5. **Arora, S. K., B. W. Ritchings, E. C. Almira, S. Lory, and R. Ramphal.** 1997. A transcriptional activator, FleQ, regulates mucin adhesion and flagellar gene expression in *Pseudomonas aeruginosa* in a cascade manner. J. Bacteriol. **179:**5574-5581.

6. **Belas, M. R., and R. R. Colwell.** 1982. Absorption kinetics of laterally and polarly flagellated *Vibrio*. J. Bacteriol. **151:**1568-1580.

7. **Boyd, A., and A. M. Chakrabarty.** 1994. Role of alginate lyase in cell detachment of *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. **60:**2355-2359.

8. **Boyd, A., and A. M. Chakrabarty.** 1995. *Pseudomonas aeruginosa* biofilms: role of the alginate exopolysaccharide. J. Indus. Microbiol. **15:**162-168.

9. **Brinton, C. C., jr.** 1965. The structure, function, synthesis and genetic control of bacterial pili and molecular model for DNA and RNA transport in gram negative bacteria. Transactions of the New York Academy of Sciences. **27:**1003-1054.

10. **Chesnokova, O., J. B. Coutinho, I. H. Khan, M. S. Mikhail, and C. I. Kado.** 1997. Characterization of flagella genes of *Agrobacterium tumefaciens*, and the effect of a bald strain on virulence. Mol. Microbiol. **23:**579-590.

11. **Christensen, G. D., W. A. Simpson, J. J. Younger, L. M. Baddour, F. F. Barrett, D. M. Melton, and E. H. Beachey.** 1985. Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of Staphylococci to medical devices. J. Clin. Microbiol. **22:**996-1006.

12. **Cockerill III, F., G. Beebakhee, R. Soni, and P. Sherman.** 1996. Polysaccharide side chains are not required for attaching and effacing adhesion of *Escherichia coli* O157:H7. Infect. Immun. **64:**3196-3200.

13. **Collinson, S. K., L. Emody, K. H. Muller, T. Trust, and W. M. Kay.** 1991. Purification and characterization of thin, aggregative fimbriae from *Salmonella enteriditis*. J. Bacteriol. **173:**4773-4781.

14. **Costerton, J. W., K. –J. Cheng, G. G. Geesey, T. I. Ladd, J. C. Nickel, M. Dasgupta, and T. J. Marrie.** 1987. Bacterial biofilms in nature and disease. Ann. Rev. Microbiol. **41:**435-464.

15. **Costerton, J. W.** 1995. Overview of microbial biofilms. J. Indus. Microbiol. **15:**137-140.

16. **Coughlan, M. P., and M. P. J. Klerstan.** 1988. Preparation and applications of immobilized microorganisms: a survey of recent reports. J. Microbiol. Methods. **8:** 51-90.

17. **Davies, D. G., A. M. Chakrabarty, and G. G. Geesey.** 1993. Exopolysaccharide production in biofilms: Substratum activation of alginate gene expression by *Pseudomonas aeruginosa*. **59:**1181-1186.

18. **Davies, D. G., and G. G. Geesey.** 1995. Regulation of alginate biosynthesis gene *algC* in *Pseudomonas aeruginosa* during biofilm development in continuous culture. Appl. Environ. Microbiol. **61:**860-867.

19. **DeFlaun, M. F., B. M. Marshall, E. P. Kulle, and S. B. Levy.** 1994. Tn*5* insertion mutants of *Pseudomonas fluorescens* defective in adhesion to soil and seeds. Appl. Environ. Microbiol. **60:**2637-2642.

20. **DeFlaun, M. F., A. Tanzer, A. L. McAteer, B. Marshall, and S. B. Levy.** 1990. Development of an adhesion assay and characterization of an adhesion-deficient mutant of *Pseudomonas fluorescens*. Appl. Environ. Microbiol. **56:**112-119.

21. **Deretic, V., M. J. Schurr, J. C. Boucher, and D. W. Martin.** 1994. Conversion of *Pseudomonas aeruginosa* to mucoidy in cystic fibrosis: Environmental stress and regulation of bacterial virulence by alternative sigma factors. J. Bacteriol. **176:**2773-2780.

22. **Doig, P., N. R. Smith, T. Todd, and R. T. Irvin.** 1987. Characterization of the binding of *Pseudomonas aeruginosa* alginate to human epithelial cells. Infect. Immun. **55:**1517-1522.

23. **Duguid, J. P., I. W. Smith, G. Dempster, and P. N. Edmunds.** 1955. Non-flagellar filamentous appendages ('fimbriae') and haemagglutinating activity in *Bacterium coli*. J. Pathol. Bacteriol. **70:**335-348.

24. **Eaton, K. A., D. R. Morgan, and S. Krakowka.** 1992. Motility as a factor in the colonization of gnotobiotic piglets by *Helicobacter pylori*. J. Med. Microbiol. **37:**123-127.

25. **Favre-Bonte, S., A. Darfeuille-Michaud, and C. Forestier.** 1995. Aggregative adherence of *Klebsiella pneumoniae* to human intestine-407 cells. Infect. Immun. **63:**1318-1328.

26. **Finlay, B. B., and S. Falkow.** 1997. Common themes in microbial pathogenicity revisited. Microbiol. Mol. Biol. Rev. **61:**136-169.

27. **Graf, J., P. V. Dunlap, and E. G. Ruby.** 1994. Effect of transposon-induced motility mutations on colonization of the host light organ by *Vibrio fischeri*. J. Bacteriol. **176:**6986-6991.

28. **Gygi, D., M. J. Bailey, C. Allison, and C. Hughes.** 1995. Requiremment of FlhA in flagella assembly and swarm-cell differentiation by *Proteus mirabilis*. Mol. Microbiol. **15:**761-769.

29. **Harber, M. J., R. Mackenzie, and A. W. Asscher.** 1983. A rapid bioluminescence method for quantifying bacterial adhesion to polystyrene. J. Gen. Microbiol. **129:**621-632.

30. **Heilmann, C., C. Gerke, F. Perdreau-Remington, and F. Götz.** 1996. Characterization of Tn*917* insertion mutants of *Staphylococcus epidermidis* affected in biofilm formation. Infect. Immun. **64:**277-282.

31. **Heilmann, C., O. Schweitzer, C. Gerke, N. Vanittanakom, D. Mack, and F. Götz.** 1996. Molecular basis of intercellular adhesion in the biofilm-forming *Staphylococcus epidermidis*. Mol. Microbiol. **20:**1083-1091.

32. **Hermansson, M., S. Kjelleberg, T. K. Korhonen, and T. A. Stenstrom.** 1982. Hydrophobic and electrostatic characterization of surface structures of bacteria and its relationship to adhesion at an air-water surface. Arch. Microbiol. **131:**308-312.

33. **Hogt, A. H., J. Dankert, C. E. Hulstaert, and J. Feijen.** 1986. Cell surface characteristics of coagulase-negative staphylococci and their adherence to fluorinated poly(ethylenepropylene). Infect. Immun. **51:** 294-301.

34. **Holst, O., A. J. Ulmer, H. Brade, H. D. Flad, and E. Th. Rietschel.** 1996. Biochemistry and cell biology of bacterial endotoxins. FEMS Immun. Med. Microbiol. **16:**83-104.

35. **Hulse, M. L., S. Smith, E. Y. Chi, A. Pham, and C. E. Rubens.** 1993. Effect of type III group B Streptococcal capsular polysaccharide on invasion of respiratory epithelial cells. Infect. Immun. **61:**4835-4841.

36. **Hultgren, S. J., S. Abraham, M. Caparon, P. Falk, J. W. St Geme III, and S. Normark.** 1993. Pilus and nonpilus bacterial adhesins: Assembly and function in cell recognition. Cell. **73:**887-901.

37. **Hultgren, S. J., C. H. Jones, and S. Normark.** 1996. Bacterial adhesins and their assembly, p. 2730-2756. In In F. C. Neidhart. *Escherichia coli* and *Salmonella typhimurium*: Cellular and molecular biology. ASM press, Washington, D. C.

38. **Hussain, M., M. Herrmann, C. van Eiff, F. Perdreau-Remington, and G. Peters.** 1997. A 140-Kilodalton extracellular protein is essential for the accumulation of *Staphylococcus epidermidis* strains on surfaces. Infect. Immun. **65:**519-524.

39. **Jacques, M., M. Kobisch, M. Bélanger, and F. Dugal.** 1993. Virulence of capsulated and noncapsulated isolated of *Pasteurella multocida* and their adherence to porcine respiratory track cells and mucus. Infect. Immun. **61:**4785-4792.

40. **Jansen, B., J. Beuth, A. Pronzondo-Mordarska, S. Jansen, H. L. Ko, and G. Pulverer.** 1992. Adhesion of fimbriated and non-fimbriaeted *Klebsiella* strains to synthetic polymers. Zbl. Bakt. **276:**205-212.

41. **Kjelleberg, S.** 1984. Adhesion to inanimate surfaces. In K. C. Marshall (ed). Microbial adhesion and aggregation. Dahlem Konferenzen. Springer, Berlin.

42. **Klemm, P.** (ed.). 1994. Fimbriae: Adhesion, genetics, biogenesis. and vaccines. CRC Press, Ann Arbor, Mich.

43. **Klemm, P.** 1994. Type 1 fimbriae of *Escherichia coli*, p. 9-26. In P. Klemm (ed.), Fimbriae: Adhesion, genetics, biogenesis. and vaccines. CRC Press, Ann Arbor, Mich.

44. **Kogure, K., E. Ikemoto, and H. Morisaki.** 1998. Attachment of *Vibrio alginolyticus* to glass surfaces is dependent on swimming speed. J. Bacteriol. **180:**932-937.

45. **Korber, D. R., J. R. Lawrence, and D. E. Caldwell.** 1994. Effect of motility on surface colonization and reproductive success of *Pseudomonas fluorescens* in dual-dilution continuous culture and batch culture systems. Appl. Environ. Microbiol. **60:**1421-1429.

46. **Krogfelt, K. A., H. Bergmans, and P. Klemm.** 1990. Direct evidence that the FimH protein is the mannose specific adhesin of *Escherichia coli* type 1 fimbriae. Infect. Immun. **58:**1995-1998.

47. **Licht, T. R., K. A. Krogfelt, P. S. Cohen, L. K. Poulsen, J. Urbance, and S. Molin.** 1996. Role of lipopolysaccharide in colonization of the mouse intestine by *Salmonella typhimurium* studied by in situ hybridization. Infect. Immun. **64:**3811-3817.

48. **Ljungh, A., A. P. Moran, and T. Wadström.** 1996. Interactions of bacterial adhesins with extracellular matrix and plasma proteins: pathogenic implications and therapeutic possibilities. FEMS Immun. Med. Microbiol. **16:**117-126.

49. **Lockman, H. A., and R. Curtiss III.** 1992. Virulence of non-type 1-fimbriated and nonfimbriated nonflagellated *Salmonalla typhimurium* mutants in murine typhoid fever. Infect. Immun. **60:**491-496.

50. **Mack, D., M. Nedelmann, A. Krokotsch, A. Schwarzkopf, J. Heesemann, and R. Laufs.** 1994. Characteriazation of transposon mutants of biofilm producing *Staphylococcus epidermidis* impaired in the accumulative phase of biofilm production: Genetic identification of a hexosamine-containing polysaccharide intercellular adhesin. Infect. Immun. **62:**3244-3253.

51. **Macnab, R.M.** 1996. Flagella and motility, p. 123-145. In F. C. Neidhart. *Escherichia coli* and *salmonella Typhimurium*: Cellular and molecular biology. ASM Press, Washington, D. C.

52. **Magnusson, K. E., and G. Johansson.** 1977. Probing the surface of *Salmonella typhimurium* and *Salmonella minnesota* SR and R bacteria by aqueous biphasic partitioning in systems containing hydrophobic and charge polymers. FEMS Microbiol. Lett. **2:**225-228.

53. **Mai, G. T., J. G. McCormack, W. K. Seow, G. B. Pier, L. A. Jackson, and Y. H. thong.** 1993. Inhibition of adherence of mucoid *Pseudomonas aeruginosa* by alginase, specific monoclonal antibodies, and antibiotics. Infect. Immun. **61:**4338-4343.

54. **Marcus, H., and N. R. Baker.** 1985. Quantitation of adherence of mucoid and nonmucoid *Pseudomonas aeruginosa* to hamster tracheal epithelium. Infect. Immun. **47:**723-729.

55. **McCormick, B. A., B. A. Stocker, D. C. Laux, and P. S. Cohen.** 1988. Roles of motility, chemotaxis, and penetration through and growth in intestinal mucus in the ability of an avirulent strain of *Salmonella typhimurium* to colonize the large intestine of streptomycin-treated mice. Infect. Immun. **56:**2209-2217.

56. **Merino, S., X. Rubires, A. Aguilar, and J. M. Tomás.** 1997. The role of flagella and motility in the adherence and invasion to fish cell lines by *Aeromonas hydrophila* serogroup O:34 strains. FEMS Microbiol. Lett. **151:**213-217.

57. **Merker, R. J., and J. Smit.** 1988. Characterization of the adhesive holdfast of marine and freshwater caulobacters. Appl. Environ. Microbiol. **54:**2078-2085.

58. **Missiakas, D., J. M. Betton, and S. Raina.** 1996. New components of protein folding in extracytoplasmic compartments of *Escherichia coli* SurA, FkpA and Skp/OmpH. Mol. Microbiol. **21:**871-884.

59. **Mobley, H. L. T., R. Belas, V. Lockatell, G. Chippendale, A. T. Trifillis, D. E. Johnson, and J. W. Warren.** 1996. Construction of a flagellum-negative mutant of *Proteus mirabilis*: Effect on internalization by human renal epithelial cells and virulence in a mouse model ascending urinary track infection. Infect. Immun. **64:**5332-5340.

60. **Moens, S., and J. Vanderleyden.** 1996. Functions of bacterial flagella. Critical Rev. Microbiol. **22:**67-100.

61. **Mol, O., and B. Oudega.** 1996. Molecular and structural aspects of fimbriae biosynthesis and assembly in *Escherichia coli*. FEMS Microbiol. Rev. **19:**25-52.

62. **Moran, A. P., M. M. Prendergast, and B. J. Appelmelk.** 1996. Molecular mimicry of host structures of bacterial lipopolysaccharides and its contribution to disease. FEMS Immun. Med. Microbiol. **16:**105-115.

63. **Mulholland, V., J. C. D. Hinton, J. Sidebotham, I. K. Toth, L. J. Hyman, M. C. M Pérombelon, P. J. Reeves, and G. P. C. Salmond.** 1993. A pleiotropic reduced virulence (Rvi$^-$) mutant of *Erwinia carotovora* subspecies *atroseptica* is defective in flagella assembly proteins that are conserved in plant and animal bacterial pathogens. Mol. Microbiol. **9:**343-356.

64. **Nevola, J. J., D. C. Laux, and P. S. Cohen.** 1987. In vivo colonization of the mouse large intestine and in vitro penetration of intestinal mucus by an avirulent smooth strain of *Salmonella typhimurium* and its lipopolisaccharide-deficient mutant. Infect. Immun. **55:**2884-2890.

65. **Olsen, A., A. Jonsson, and S. Normark.** 1989. Fibronectin mediated by a novel class of surface organelles on *Escherichia coli*. Nature. **338:**652-655.

66. **Ong, C. J., M. L. Y. Wong, and J. Smit.** 1990. Attachment of the adhesive holdfast organelle to the cellular stalk of *Caulobacter crescentus*. J. Bacteriol. **172:**1448-1456.

67. **Parker, C. T., A. W. Kloser, C. A. Schnaitman, M. A. Stein, S. Gottesman, and B.W. Gibson.** 1992. Role of the *rfaG* and *rfaP* in determining the lipopolysaccharide core structure and cell surface properties of *Escherichia coli* K-12. J. Bacteriol. **174:**2525-38.

68. **Pierson, D. E.** 1994. Mutations affecting lipopolysaccharide enhance ail-mediated entry of *Yersinia enterocolitica* into mammalian cells. J. Bacteriol. **176:**4043-4051.

69. **Pilipcinec, E., T. T. Huisman, P. T. J. Willemsen, B. J. Appelmelk, F. K. de Graaf, and B. Oudega.** 1994. Identification by Tn*10* transposon mutagenesis of host factors involved in the biosynthesis of K99 fimbriae of *Escherichia coli*: Effect of LPS core mutations. FEMS Microbiol. Lett. **123:**201-206.

70. **Pringle, J. H., and M. Fletcher.** 1983. Influence of substratum wettability on attachment of freshwater bacteria to solid surfaces. Appl. Environ. Microbiol. **45:**811-817.

71. **Pringle, J. H., M. Fletcher, and D. C. Ellwood.** 1983. Selection of attachment mutants during the continuous culture of *Pseudomonas fluorescens* and relationship between attachment ability and surface composition. J. Gen. Microbiol. **129:**2557-2569.

72. **Quintero, E. J., and R. M. Weiner.** 1995. Evidence for the adhesive function of the exopolysaccharide of *Hyphomonas* strain MHS-3 in its attachment to surfaces. Appl. Environ. Microbiol. **61:**1897-1903.

73. **Quintero, E. J., and R. M. Weiner.** 1995. Physical and chemical characterization of the polysaccharide capsule of the marine bacterium, *Hyphomonas* strain MHS-3. J. Indus. Microbiol. **15:**347-351.

74. **Raetz, C. R.** 1996. Bacterial lipopolysaccharides: a remarkable family of bioactive macroamphiphiles, p. 1035-1063. In F. C. Neidhart. *Escherichia coli* and *salmonella Typhimurium*: Cellular and molecular biology. ASM Press, Washington, D. C.

75. **Ramphal, R., and G. B. Pier.** 1985. Role of *Pseudomonas aeruginosa* mucoid exopolysaccharide in adherence to tracheal cells. Infect. Immun. **47:**1-4.

76. **Reid, G., H. C. van der Mei, C. Tieszer, and H. J. Busscher.** 1996. Uropathogenic *Escherichia coli* adhere to urinary catheters without using fimbriae. FEMS Immun. Med. Microbiol. **16:**159-162.

77. **Roberts, I. S.** 1996. The biochemistry and genetics of capsular polysaccharide production in bacteria. Annu. Rev. Microbiol. **50:**285-315.

78. **Rosenberg, M., E. A. Bayer, J. Delarea, and E. Rosenberg.** 1982. Role of thin fimbriae in adherence and growth of *Actinobacter caloaceticus* on hexadecane. Appl. Environ. Microbiol. **44:**929-937.

79. **Rosenberg, M., D. Gutnick, and E. Rosenberg.** 1980. Adherence of bacteria to hydrocarbons: A simple method for measuring cell-surface hydrophobicity. FEMS Microbiol. Lett. **9:**29-33.

80. **Rosenberg, E., N. Kaplan, O. Pines, M. Rosenberg, and D. Gutnick.** 1983. Capsular polysaccharides interfere with adherence of *Acinetobacter calcoaceticus* to hydrocarbon. FEMS Microbiol. Lett. **17:**157-160.

81. **Runnels, P. L., and H. W. Moon.** 1984. Capsule reduces adherence of enterotoxigenic *Escherichia coli* to isolated intestinal epithelial cells of pigs. Infect. Immun. **45:**737-740.

82. **Savelkoul, P. H. M., D. P. G. de Kerf, R. J. Willems, F. R. Mooi, B. A. M. van der Zeijst, and W. Gaastra.** 1996. Characterization of the *fim2* and *fim3* fimbrial subunit genes of *Bordetella bronchiseptica*: Role of Fim2 and Fim3 fimbriae and flagella in adhesion. Infect. Immun. **64:**5098-5105.

83. **Schmidt, M. A.** 1994. Nonfimbrial adhesins of Escherichia coli, p. 85-96. In P. Klemm (ed.), Fimbriae: Adhesion, genetics, biogenesis. and vaccines. CRC Press, Ann Arbor, Mich.

84. **Schmitt, C. K., S. C. Darnell, and A. D. O'Brien.** 1996. The *Salmonella typhimurium flgM* gene, which encodes a negative regulator of flagella synthesis and is involved in virulence, is present and functional in other *Salmonalla* species. FEMS Microbiol. Lett. **135:**281-285.

85. **Schmitt, C. K., S. C. Darnell, V. L. Tesh, B. A. D. Stocker, and A. D. O'Brien.** 1994. Mutation of *flgM* attenuates virulence of *Salmonella typhimurium*, and mutation in *fliA* represses the attenuated phenotype. J. Bacteriol. **176:**368-377.

86. **Schnaitman, C. A., and J. D. Klena.** 1993. Genetics of lipopolysaccharide biosynthesis in enteric bacteria. Microbiol. Rev. **57:**665-682.

87. **Shea, C., L. J. Lovelace, and H. E. Smith-Somerville.** 1995. *Deleya marina* as model organism for studies of bacterial colonization and biofilm formation. J. Indus. Microbiol. **15:**290-296.

88. **Shea, C., J. W. Nunley, J. C. Williamson, and H. E. Smith-Somerville.** 1991. Comparaison of the adhesion properties of *Deleya marina* and the exopolysaccharide-defective mutant strain DMR. Appl. Environ. Microbiol. **57:**3107-3113.

89. **Silverman, M., M. R. Belas, and M. Simon.** 1984. Genetic control of bacterial adhesion, p. 95-107. In K. C. Marshall (ed.), Microbial adhesion and aggregation. Springer-Verlag, Berlin, Germany.

90. **Sokurenko, E. V., H. S. Courtney, D. E. Ohman, P. Klemm, and D. L. Hasty.** 1994. FimH family of type 1 fimbrial adhesins: Functional heterogeneity due to minor sequence variations among *fimH* genes. J. Bacteriol. **176:**748-755.

91. **Stenström, T. A., and S. Kjelleberg.** 1985. Fimbriae mediated non-specific adhesion of *Salmonella typhimurium* to mineral particles. Arch. Microbiol. **143:**6-10.

92. **St. Geme, J. W. III, and S. Falkow.** 1991. Loss of capsule expression by *Haemophilis influenzae* type b results in enhanced adherence to and invasion of epithelial cells. Infect. Immun. **59:**1325-1333.

93. **Stock, J. B., and M. G. Surette.** 1996. Chemotaxis, p. 1103-1129. In F. C. Neidhart. *Escherichia coli* and *Salmonella typhimurium*: Cellular and molecular biology. ASM Press, Washington, D. C.

94. **Tenney, M. W., and W. Stumm.** 1965. Chemical flocculation of microorganisms in biological waste treatment. J. Water Pollution Control Federation. **37:**1370-1388.

95. **Timmerman, C. P., A. Fleer, J. M. Besnier, L. De Graaf, F. Cremers, and J. Verhoef.** 1991. Characterization of a proteinaceous adhesin of *Staphylococcus epidermidis* which mediates attachment to polystyrene. Infect. Immun. **59:**4187-4192.

96. **Veenstra, G. J. C., F. F. M. Cremers, H. van Dijk, and A. Fleer.** 1996. Ultrastructural organization and regulation of a biomaterial adhesin of *Staphylococcus epidermidis*. J. Bacteriol. **178:**537-541.

97. **Vidal, O., R. Longin, C. Prigent-Combaret, C. Dorel, M. Hooreman, and P. Lejeune.** 1998. Isolation of an *Escherichia coli* K-12 mutants strain able to form biofilms on inert surfaces: Involvment of a new ompR allele that increases curli expression. J. Bacteriol. **180:**2442-2449.

98. **Weissborn, A. C., Q. Lui, M. K. Rumley, and E. P. Kennedy.** 1994. UTP:α-D-Glucose-1-Phosphate Uridylyltransferase of *Escherichia coli*: Isolation and DNA sequence of the *galU* gene and purification of the enzyme. J. Bacteriol. **176:**2611-2618.

99. **Whittaker, C. J., C. M. Klier, and P. E. Kolenbrander.** 1996. Mechanisms of adhesion by oral bacteria. Ann. Rev. Microbiol. **50:**513-552.

100. **Williams, V., and M. Fletcher.** 1996. *Pseudomonas fluorescens* adhesion and transport through porous media are affected by lipopolysaccharide composition. Appl. Environ. Microbiol. **62:**100-104.

101. **Wilson, M.** 1996. Suceptibility of oral bacterial biofilms to antimicrobial agents. J. Med. Microbiol. **44:**79-87.

102. **Wrangstadh, M., P. L. Conway, and S. Kjelleberg.** 1986. The production and release of an extracellular polysaccharide during starvation of a marine *Pseudomonas* sp. and the effect thereof on adhesion. Arch. Microbiol. **145:**220-227.

103. **Wrangstadh, M., P. L. Conway, and S. Kjelleberg.** 1989. The role of an extracellular polysaccharide produced by the marine *Pseudomonas* sp. S9 in cellular detachment during starvation. Can. J. Microbiol. **35:**309-312.

104. **Wrangstadh, M., U. Szewzyk, J. Östling, and S. Kjelleberg.** 1990. Starvation-specific formation of a peripheral exopolysaccharide by a marine *Pseudomonas* sp. strain S9. Appl. Environ. Microbiol. **56:**2065-2072.

105. **Yao, R., D. H. Burr, P. Doig, T. J. Trust, H. Niu, and P. Guerry.** 1994. Isolation of motile and non-motile insertional mutants of *Campylobacter jejuni*: the role of motility in adherence and invasion of eukaryotic cells. Mol. Microbiol. **14:**883-893.

106. **Yun, C. Y., B. Ely, and J. Smit.** 1994. Identification of genes affecting production of the adhesive holdfast of a marine caulobacter. J. Bacteriol. **176:**796-803.

107. **Ziebuhr, W., C. Hellmann, F. Götz, P. Meyer, K. Wilms, E. Straube, and J. Hacker.** 1997. Detection of the intercellular adhesion gene cluster (*ica*) and phase variation in *Staphylococcus epidermidis* blood culture strains and mucosal isolates. Infect. Immun. **65:**890-896.

108. **Zottola, E. A., and K. C. Sasahara.** 1994. Microbial biofilms in the food processing industry-Should they be a concern? Int. J. Food Microbiol. **23:**125-148.

Chapter II

A Rapid Screening Procedure to Identify Mini-Tn*10* Insertion Mutants of *Escherichia coli* K-12 with Altered Adhesion Properties

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ABSTRACT

A rapid screening procedure was developed for detection of *Escherichia coli* mutants with altered adhesion abilities using polystyrene 96 well microtiter plates as attachment surfaces. During this assay, bacterial strains grew and adhered simultaneously, and attached cells were measured after crystal violet staining. Starting with a total of 7000 W3110:Tn*10* insertion mutants of *E. coli* K-12 W3110, 50 adhesion deficient mutants were isolated which showed <40% attachment, and 22 mutants were found with an attachment of 40% to 75%. Motility assays were performed on these 72 mutants, and 34 displayed altered motility.

INTRODUCTION

The natural tendency of bacteria to adhere to surfaces and therefore to initiate biofilm formation in most oligotrophic environments can lead to a variety of economic and health problems.

Bacterial adhesion to both natural and artificial surfaces has been examined in many different fields, including material deterioration studies, human and animals diseases, and in environmental microbiology (2). Adhesion to inanimate surfaces is a complex process in which bacteria utilize several mechanisms to adhere to different surfaces under a plethora of environmental conditions. Apart from some bacterial structures for specific attachment, such as fimbriae adhesins or holdfasts, less is known about other features that play a role in bacterial adhesion to surfaces (7). In order to obtain a better understanding of the processes required for the primary step(s) of attachment and biofilm formation, genetic approaches may provide further insights. This type of study requires the production of a large number of mutants plus extensive screening for those with altered adhesion properties.

In this study, random insertion mutagenesis was performed with a mini-Tn*10* in *Escherichia coli* K-12 W3110. A rapid screening adhesion assay, based on staining of adherent cells to a polystyrene surface, was developed and used to identify non-adhesive mutants. In addition, a preliminary classification of those mutants with altered adhesion abilities was performed through an analysis of their motility.

MATERIALS AND METHODS

Bacterial strains, bacteriophage, and culture conditions

E .coli K-12 W3110 (1) was used as the parental strain for adhesion experiments and mutagenesis. *E. coli* AD87, a previously-isolated mini-Tn*10* insertion mutant of W3110 with altered adhesion properties, was used to optimize the screening method.

A random mutagenesis was performed with mini-Tn*10* (Km^r) derivative 103, located in a $P_{am}80$ λ hop phage vehicle (λNK1316). Details of the basic procedure were described by Kleckner *et al.* (8). Mini-Tn*10* insertion mutants of W3110 were selected on M9 minimal medium supplemented with glucose and kanamycin (10), for selection of prototrophic mutants.

The medium used for bacterial growth and the adhesion-screening assay was LB medium (10) supplemented with kanamycin (30 μg/ml).

Motility tests were performed on semi-solid medium, as described by Macnab (11). Results were confirmed by direct microscopic examination.

Adhesion assay

Selection of mutants with altered adhesion abilities was performed by a rapid screening method in 96 well microtiter plates. The procedure of staining adhered cells, as described by Cowan *et al.* (3), was modified by suspending cells in LB medium prior to adherence.

Briefly, single colonies of each transposon mutant were inoculated and allowed to grow for 10 hours in 5 ml of LB plus kanamycin broth at 37°C without shaking. Duplicate 20 μl samples of each culture in log phase (A_{630} of approx 0,6), plus 200 ml of LB Km broth, were placed in wells of a polystyrene microtiter plate.

Mutants were allowed to grow and adhere for 2 hours at 37°C. At the end of that period, absorption (A_{630}) of the culture was measured on a Dynatech MRX microplate reader. Unbound cells were removed by inversion of the microtiter plate, followed by vigorous tapping on absorbent paper. Subsequently, adhered cells were fixed for 30 min at 80°C.

Adhered cells were stained by addition of 220 μl of crystal violet (0,1%) for 1 min. The stain was removed by exhaustive washing with distilled water. The plates were then allowed to dry. In order to quantify adhered cells, 220μl of decolouring solution (ethanol 80% / acetone 20%) was added to each well for 15 min. The absorption of the eluted stain was measured at 590nm.

RESULTS AND DISCUSSION

Figure 1 shows the adhesion ability, versus time of inoculation into polystyrene microtiter plates, of *E. coli* W3110 and AD87. AD87 is a mini-Tn*10* insertion mutant of W3110 with reduced numbers of adhered cells when compared with the parent strain in our assay. Parent strain adhesion increased linearly versus time for up to seven hours. The decrease, and ultimate plateau found in the number of adherent cells after that period, may be due to a combination of cell number, cell age, age of the culture and available surface (5). As shown in figure 1, two hours of contact with the surface were sufficient to detect differences in adhesion properties between the wild type and AD87 strains. This period of time was chosen to screen 7000 mini-Tn*10* insertion mutants. A typical set of results is shown in Figure 2.

Fifty mini-Tn*10* insertion mutants of W3110 among a total of 7000, were found to adhere less than 40% of wild type level, while 22 mutants showed an adhesion level of 40% to 75%, when compared to the parent strain. Motility tests were performed to classify those less adhesive mutants. Of the 72 mutant clones assayed, a total of 38 clones displayed normal motility. Moreover, 33 of these clones adhered less than 40%, while 5 showed an adherence level of between 40% and 75%. The other 34 mutants showed altered motility equally divided between those that adhered less than 40% and those which showed an adherence level of between 40% and 75%.

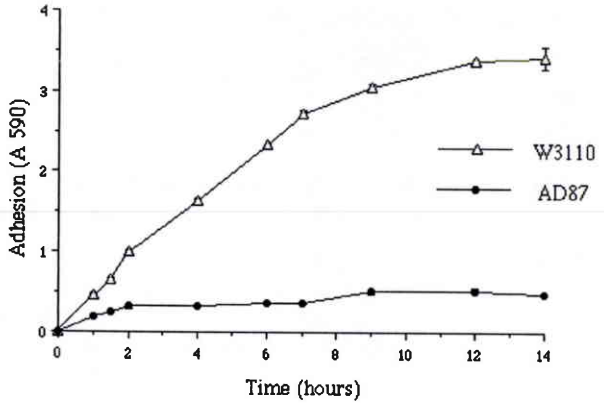

Fig. 1. Relationship between time and adhesion of two *E. coli* strains, W3110 (Δ) and AD87 (●), to polystyrene microtiter plates.

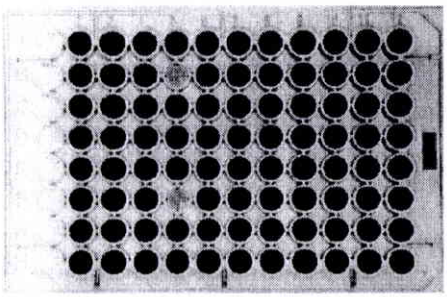

Fig. 2. Microplate assay. A typical set of results is shown. The strains in B5 and F5 displayed a reduced adhesion ability.

The large number of adhesion mutants with altered motility is in agreement with previous studies concerning the involvement of motility in adhesion. The adhesive ability of *Vibrio fischeri* suggests that the presence of flagella alone is not sufficient to colonize juvenile *Euprymna scolopes*, but functional motility is required (6). In addition Korber *et al.* (9), showed that motility conveyed a selective advantage during colonization of glass surfaces by *Pseudomonas fluorescens* strains. Furthermore, Tn5 insertion mutants of *P. fluorescens* defective in the initial stages of attachment to soil and seeds have been shown to be deficient in motility (4). In contrast, half of the mutants obtained in this study were not deficient in motility. Our results indicate that other functions, not involved in motility, play a role in *E. coli* adhesion.

The screening method used was based on adhesion of growing cells to polystyrene microtiter plates. Care must be taken to distinguish between growth-impaired mutants and adhesion mutants. It is thus important to measure the cell density in wells after the contact time, as described in Materials and Methods, and to screen the putative adhesion mutant strains at different times of adhesion, as reported in Figure 1.

In conclusion, a rapid screening method to detect bacteria with altered adhesion abilities has been developed. The microassay reported here has the advantage to require small quantities of bacteria, culture media and reagents and to be reproducible and rapid. As reported here a set of *E. coli* K-12 mini-Tn*10* insertion mutants with altered adhesion abilities has been obtained. Further investigation is ongoing to characterize genes involved.

ACKNOWLEDGEMENTS

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REFERENCES

1. **Bachmann, B. J.** 1972. Pedigrees of some mutant stains of *Escherichia coli* K-12, Bacteriol. Rev. **36:**525-557.

2. **Costerton, J. W., K. J. Cheng, G. G. Geesey, T. I. Ladd, J. C. Nickel, M. Dasgupta, and T. J. Marrie.** 1987. Bacterial biofilms in nature and disease, Ann. Rev. Microbiol. **41:**435-464.

3. **Cowan, M. M., and M. Fletcher.** 1987. Rapid screening method for detection of bacterial mutants with altered adhesion abilities, J. Microbiol. Methods **7:**241-249.

4. **DeFlaun, M. F., B. M. Marshall, E. P. Kulle, and S. B. Levy.** 1994. Tn*5* Insertion mutants of *Pseudomonas fluorescens* defective in adhesion to soil and seeds, Appl. Environ. Microbiol. **60:**2637-2642.

5. **Fletcher, M.** 1977. The effects of culture concentration and age, time, and temperature on bacterial attachment to polystyrene, Can. J. Microbiol. **23:**1-6.

6. **Graf, J., P. V. Dunlap, and E. G. Ruby.** 1994. Effect of transposon-induced motility mutations on colonization of the host light organ by *Vibrio fischeri*, J. Bacteriol. **176:**6986-6991.

7. **Kjelleberg, S.** 1984. Adhesion to inanimate surfaces, In: Microbial adhesion and aggregation, ed. K.C. Marshall, pp 52-70. Dahlem Konferenzen. Berlin: Springer-Verlag.

8. **Kleckner, N., J. Blender, and S. Gottesman.** 1991. Uses of transposons with emphasis on Tn*10*, Methods Enzym. **204:**139-180.

9. **Korber, D. R., J. R. Lawrence, and D. E. Caldwell.** 1994. Effect of motility on surface colonization and reproductive success of *Pseudomonas fluorescens* in dual-dilution continuous culture and batch culture systems, Appl. Environ. Microbiol. **60:**1421-1429.

10. **Macnab, R. M.** 1986. Proton-driven bacterial flagellar motor, Methods Enzym. **125:**563-581.

11. **Miller, J. H.** 1992. *A short course in bacterial genetics; A laboratory manual and handbook for E. coli and related bacteria*. Cold Spring Harbor, NY, USA. Cold Spring Harbor Laboratory.

Chapter III

The Role of Flagella and Type 1 Fimbriae in *Escherichia coli* K-12 Adhesion to Inanimate Surface

ABSTRACT

A mini-Tn*10* random mutagenesis was carried out in *E. coli* K-12 W3110, and mutants with altered adhesion abilities to polystyrene surface were isolated (P. Genevaux, S. Muller, and P. Bauda, FEMS Microbiol. Lett. 142:27-30, 1996). The precise location of the transposon in the affected genes of the adhesion mutants was determined using inverse PCR and DNA sequence analysis. Twenty-five insertions were found to be located in the *fim* operon encoding type 1 fimbriae, and twelve insertions were found in genes involved in flagella biosynthesis. The adhesion properties, growth, type 1 fimbriae synthesis, motility capability, and flagella morphology of the mutants were determined, and important correlations between the amount of fimbriae, adhesion, and growth were observed. All the *fim* insertion mutants did not produce fimbriae, exhibited a weak attachment to polystyrene, and grew poorly in the conditions used. In addition, adhesion of the parent strain was reduced to the level of the *fim* mutants when mannose was added to the incubation medium. The observed mannose-sensitive adhesion demonstrated a direct involvement of the type 1 fimbriae specific adhesin FimH in adherence to polystyrene.

Two groups of adhesion mutants were observed among the mutants affected in flagella biosynthesis. The first group of mutants (insertions located in class 2 genes of flagella biosynthesis) exhibited poor adhesion, grew poorly, were non-motile, non-flagellated, and produced about 30% of the amount of fimbriae when compared to the wild-type cells. The second group (insertion in the promoter region of the class 3 *fliC* gene) adhered and grew better than the first group, produced abnormal flagella, had dysfunctional motility and about 70% of the amount of fimbriae as compared to the parent strain. The loss of adhesion was mainly due to the dysfunctional motility and to the attenuated fimbriation enhanced by the flagella mutations.

INTRODUCTION

Bacteria have the inherent tendency to interact and grow in association with surfaces (7, 8). The study of bacterial adhesion to biological as well as non-biological surfaces has implications in many different fields, including microbial ecology, human and animal pathology, and industrial microbiology (7). Adhesion to inanimate surfaces is a complex process in which bacteria utilize several mechanisms to adhere to various surfaces under a plethora of environmental conditions. Both polysaccharides and membrane proteins were found to play a role in this phenomenon. Some exopolysaccharides appear to be synthesized only

when bacteria are attached to surfaces (4, 43). Lipopolysaccharides were also found in some cases to influence adhesion (46). Flagella have often been shown to play an important role during microbial colonization events by affecting initial attachment to the surface. Functional flagella may confer selective advantages during surface colonization (9, 10, 16, 28). Bacterial fimbriae, extensively studied for specific adhesion to biological surfaces, were identified in some cases as active participants in solid surface colonization (19, 39, 44). Questions of which membrane structures are required for a given surface, and how these adhesives interact with each other have not been completely elucidated to date.

The well-characterized bacterium *Escherichia coli* K-12 was used as model to identify genes and cell surface components associated with adhesion to a polystyrene surface. Random mutagenesis with a mini-Tn*10* was carried out and mutants with changes in binding properties were isolated (15). The position of the transposon in the mutated genes was determined using inverse PCR and DNA sequence analysis. Here, we report that mutations located in the *fim* operon, encoding for type 1 fimbriae, and in flagella genes, affect adhesion. The adhesion properties of these mutants were determined, and the alterations in growth, fimbriae and flagella biosynthesis associated with these mutations were analyzed. The role of type 1 fimbriae and flagella in adhesion to non-biological surfaces, and the effect of flagella mutations on fimbriae biosynthesis, are described.

MATERIALS AND METHODS

Bacterial strains, bacteriophage, and culture conditions

The *E. coli* K-12 strains used in this study are described in Table 1. Strains constructed for this work are mini-Tn*10* insertion mutants of *E. coli* K-12 W3110 with altered adhesion properties as described previously (15). Bacterial growth and adhesion experiments were carried out in static LB medium at 37°C supplemented with kanamycin (30 μg/ml) when necessary (35). To check for unique site mutations in the W3110::Tn*10* adhesion mutants, the mutation was transferred back into the parent strain W3110 using the bacteriophage P1 *vir* as described by Miller (35).

DNA techniques, inverse PCR methodology and nucleotide sequencing

All standard DNA procedures were carried out essentially as described by Sambrook *et al.* (41). Restriction endonucleases were used according to the manufacturer's specifications

(New England Biolabs).

For localization of the mini-Tn*10* insertion mutations in the *E. coli* chromosome, inverse PCR was carried out. Two oligonucleotides annealing closely together at the IS*903* termini of the mini-Tn*10* derivative 103 (Km^r) (22) were prepared (primer 1: 5'-TTA CAC TGA TGA ATG TTC CG-3', primer 2: 5'-GTC AGC CTG AAT ACG CGT-3'). Chromosomal DNA of the mutant strains was isolated and digested with either *Ava* II or *Hae* II. These restriction enzymes did not digest the region between the 3' end of primer II and the end of IS*10*. The resultant DNA restriction fragments were then circularized. Subsequently, PCR using the circular fragments was carried out with 50 pmol of each primer. The reactions were run for 1 min at 96°C and thereafter for 30 cycles each composed of 10 sec at 96°C, 30 sec at 55°C, and 2 min and 30 sec at 65°C. The PCR products obtained were then extracted from agarose gels by centrifugation through blotting paper (45) and purified by phenol chloroform isoamyl alcohol (50:48:2 by volume) extraction. Nucleotide sequencing was carried out by using the Thermo Sequenase dye-terminator cycle sequencing kit from Amersham, with primer I as sequencing primer and double stranded PCR DNA products as template. Sequence analyses were carried out using the 373A semi-automated DNA sequencer of Applied Biosystems/Perkin-Elmer. The obtained DNA sequences were checked for homology in the Genbank library.

Adhesion experiments

Adhesion experiments were carried out using polystyrene microtiter plates as previously described (15). Briefly, 20 µl of a cell culture with a culture turbidity of 0.70 at 580 nm (A_{580}=0.70) in static liquid LB medium supplemented with kanamycin were placed in a polystyrene microtiter plate containing 200 µl of fresh LB kanamycin broth. Cells were then allowed to grow and adhere for 2 h; 5h; 8.5 h; 13 h; and 20 h at 37°C. The optical density of the culture in the well was measured (A_{580}), and the unbound cells were removed. Adhered cells were fixed 30 min at 80°C and quantified by crystal violet staining.

Table 1: Bacterial strains used in this study.

Strains	Genotype[a]	Phenotype		GenBank accession number	Source or reference
		Haemagglutination[b]	Motility[c]		
HB101	F⁻ *lacY1 recA13 Δfim*	-	NT	-	(5)
MG1655	F⁻ λ⁻ Fim+	NT	+	-	(3)
AAEC143	MG1655; *fimE*	NT	+	-	(3)
W3110	F⁻ λ⁻ IN(*rrnD-rrnE*)1 *rph-1*	A	+	-	(2)
***fim* mutants**					
BGF2	*fimB* 232328	-	+	U14003	this study
BGF3	*fimA* 233526	-	+	"	"
BGF4	*fimA* 234206	-	+	"	"
BGF5	*fimA* 234438	-	+	"	"
BGF6	*fimA* 234446	-	+	"	"
BGF8	*fimA* 234488	-	+	"	"
BGF9	*fimI* 234952	-	+	"	"
BGF10	*fimC* 235393	-	+	"	"
BGF11	*fimC* 235594	-	+	"	"
BGF12	*fimC* 235735	-	+	"	"
BGF13	*fimC* 235765	-	+	"	"
BGF15	*fimD* 235974	-	+	"	"
BGF16	*fimD* 236281	-	+	"	"
BGF18	*fimD* 236327	-	+	"	"
BGF19	*fimD* 236363	-	+	"	"
BGF20	*fimD* 236463	-	+	"	"
BGF21	*fimD* 236540	-	+	"	"
BGF22	*fimD* 236772	-	+	"	"
BGF23	*fimD* 236798	-	+	"	"
BGF25	*fimD* 237663	-	+	"	"
BGF28	*fimD* 237720	-	+	"	"
BGF30	*fimD* 238003	-	+	"	"
BGF31	*fimD* 238353	-	+	"	"
BGF32	*fimH* 240057	-	+	"	"
BGF33	*fimH* 239735	-	+	"	"
flagella mutants					
BGM1	*fliR* 1437	C	-	L22182	"
BGM2	*fliR* 1943	C	-	"	"
BGM3	*fliR* 1947	C	-	"	"
BGM5	*fliR* 207	C	-	"	"
BGM6	*fliP* 650	C	-	"	"
BGM8	*fliM* 480	C	-	M12784	"
BGM9	*fliG* 75	C	-	U46011	"
BGM10	*fliG* 795	C	-	"	"
BGM11	*fliC* 685	B	+/-	X17440	"
BGM13	*fliI* 411	C	-	L49147	"
BGM14	*flgA* 519	C	-	D25292	"
BGM15	*flgG* 1125	C	-	X52094	"

[a] For all the W3110 derivative strains, the number of the mutation refers to the nucleotide position of the Tn*10* in the nucleotide sequence of the corresponding GenBank accession number.

[b] Average time value, based on triplicate measurements, until agglutination occurred; A: 30 sec, B: 65 sec, C:

between 156 and 181 sec, -: no agglutination, NT: non tested.
[c] Determined by the production of halos on soft agar plates and by observation under phase contrast microscopy; +: wild-type, +/-: dysfunctional motility, -: no motility.

Spot blot analysis

The capacity of bacteria to produce type 1 fimbriae was estimated using spot blot analysis. Single colonies of each strain were grown at 37°C in static LB medium to an A_{580} of 0.70, centrifuged (30 sec at 7 000 x *g*) and resuspended in phosphate-buffered saline (136.9mM NaCl, 2.7 mM KCl, 10.0 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7.2).

Serial dilutions of cell aliquots were spotted on 0.2 µm nitrocellulose membrane paper (Schleicher & Schuell), using the Bio-Dot apparatus from Bio-Rad. Next, the membranes were incubated with polyclonal rabbit antibodies directed against type 1 fimbriae, kindly provided by Maryvonne Dho-Moulin (INRA. Tours, France). Blots were then incubated with goat anti-Rabbit conjugated to horse radish-peroxidase as secondary antibodies. Detection was carried out using the ECL kit from Amersham. To quantify the production of type 1 fimbriae, spot densities were analyzed with the NIH-image program version 1.56.

Haemagglutination

The capacity of bacterial cells to express a D-mannose-binding phenotype was assayed by their ability to agglutinate rabbit erythrocytes as described by Klemm *et al.* (27). Briefly, 50 µl of an aliquot of bacterial cells in phosphate-buffered saline (A_{580}=10.0) plus 50 µl of 5% rabbit erythrocytes in KRT (128.3 mM NaCl, 5.1 mM KCl, 1.3 mM $MgSO_4.7H_2O$, 2.7 mM $CaCl_2.2H_2O$, 10.0 mM Tris-HCl, pH 7.4) were mixed on glass slides and the time until agglutination occurred was monitored.

Motility assay

Motility tests were carried out by applying 1 µl of cell culture (A_{580}=0.7) on soft agar plates (1 % Tryptone, 0.5 % Nacl, 0.35 % agar). After 6 h of incubation at 37°C, the diameters of growth zones were measured. These analyses were complemented by direct microscopic examination.

Electron microscopy

Electron microscopic analysis was carried out as follows: bacterial cell cultures

(A_{580}=0.7) were gently washed in phosphate-buffered saline and concentrated to an A_{580} of 3.0. Cell aliquots were placed on a nickel Formvar-coated grid for 2 min, blotted dry and stained for 10 seconds with a 1% tungsten solution (pH 7.0).

RESULTS

Isolation of *fim* mutations

A total of 7000 *E. coli* K-12 W3110::Tn*10* insertion mutants were screened for their ability to adhere to a polystyrene surface as previously described (15). Twenty-five transposon mutants that expressed a reduced adhesion had insertions in the *fim* operon responsible for type 1 fimbriation, at 97.6 min on the *E. coli* K-12 chromosome (Table 1) (25). The precise location of the mini-Tn*10* transposon in the *fim* cluster was determined by inverse PCR, nucleotide sequencing, and computer analysis, and is shown in Table 1. The majority of these transposon insertions were found to be in the *fimD* gene, which encodes an outer membrane protein of 97 kDa, involved as molecular usher in the biogenesis of the type 1 fimbriae (26). Additionally, four mutations were found in the *fimC* gene encoding the periplasmic chaperone FimC involved in binding and folding of fimbrial subunits (24). Four other transposon insertions were detected in the gene encoding the major subunit FimA, and one was found in the *fimI* gene, encoding a FimA homologue of unknown function (25). Two insertions were located in the *fimH* gene encoding the minor fimbrial subunits FimH, the adhesin molecule of type 1 fimbriae which binds specifically to α-D-mannopyranosyl receptors (18, 30). Finally, one mutant was found with an insertion in the *fimB* regulatory gene, whose product promotes the inversion of the invertible sequence containing the *fimA* promoter (23).

Adhesion, growth and fimbriation of the *fim* insertion mutants

The *fim* mutants were analyzed for their adhesion properties. Adhesion and cell culture density were measured at various times after inoculation into the microplate. All of the *fim* mutants were strongly and identically affected in their ability to adhere to polystyrene. Figure 1 shows, as a typical result, the adhesion ability of the parent strain W3110 and the mini-Tn*10* insertion BGF4 *fimA*.

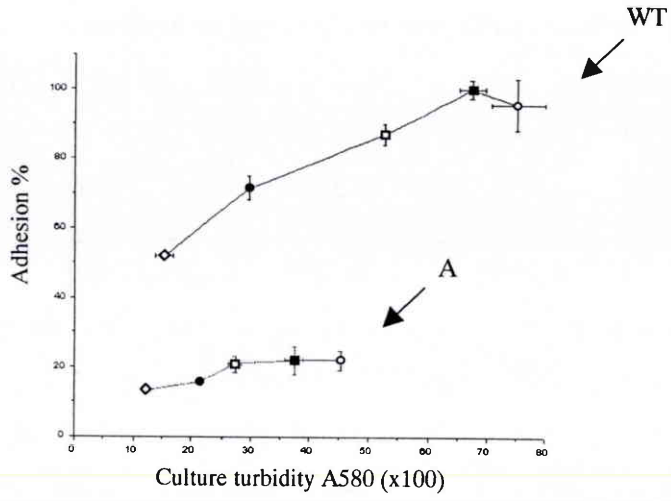

Fig. 1. Adhesion to polystyrene and growth of *fim* mutants: WT, W3110 parent strain; A, BGF4 *fimA*. Adhesion (%) and culture density (A_{580}) were measured at 2 h (◇), 5 h (●), 8.5 h (□), 13 h (■), and 20 h (○) after inoculation into the microplate. Adhesion is represented as percentage of adhesion compared to the wild type maximal adhesion. Error bars indicate the standard deviation based on triplicate measurements.

The parent strain adhesion increased linearly for up to 13 h. After that period, a decrease due to cells entering stationary phase was observed (11, 14). Addition of 2.5% D-mannose (a specific carbohydrate receptor for the type 1 fimbriae adhesin) to the incubation medium during the adhesion assay restored the wild-type adhesion properties to the *fim* mutants level (data not shown). Adhesion of the strain BGF4 *fimA* (representative of the 25 *fim* mutants identified) increased slowly during the first 8.5 h and then reached an ultimate plateau (about 20% of the wild-type maximal adhesion). In addition to the considerably affected adhesion, the growth of the *fim* adhesion mutants was strongly reduced as compared to the parent strain (see Discussion) (Fig. 1).

Fimbriae production of these *fim* insertion mutants was estimated by spot blot analysis with polyclonal antibodies raised against type 1 fimbriae. The strain *E. coli* K-12 HB101 Δ*fim* served as a negative control. All the *fim* insertion mutants identified behaved like strain HB101 Δ*fim*, and showed a complete lack of fimbriation, and adhesion at minimal levels. To determine the capacity of the *fim* mutants to express a D-mannose-binding phenotype and therefore to produce the adhesin FimH at the surface of the cell, haemagglutination tests with rabbit erythrocytes were carried out. The parent strain (W3110) agglutinated rabbit erythrocytes after 30 sec of contact, whereas the *fim* mutants did not show any agglutination after 15 minutes

(Table 1). These results show no FimH functionality in any of the mutants.

Fimbriae overproduction and adhesion

To demonstrate the effect of an overexpression of type 1 fimbriae to the attachment, strain MG1655 (*fim*+) and its derivative AAEC143 *fimE*, which produces more fimbriated cells under these conditions (3), used were tested for adhesion to polystyrene microtiter plates after 2 and 5 h of incubation. A culture of strain AAEC143 contains more fimbriated cells when cultivated in static broth culture (3). This *fimE* mutant strain showed a significant increase in adhesion abilities when compared to the parent strain MG1665 (Fig. 2).

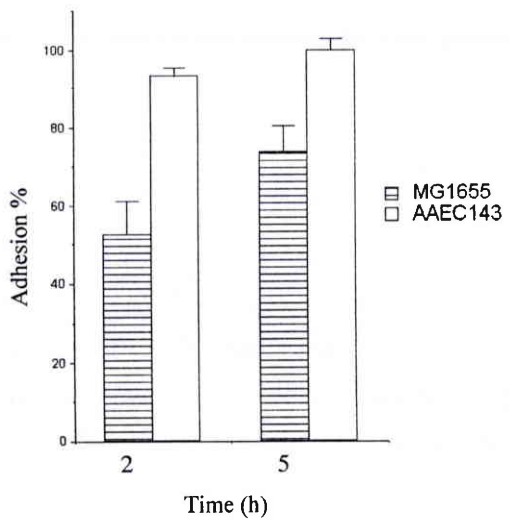

Fig. 2. Relationship between time and adhesion to polystyrene microtiter plates, of AAEC143 *fimE* and the parent strain MG1655. Error bars signify standard deviation based on triplicate measurements. The value 100% corresponded to the maximal adhesion of the strain AAEC143 *fimE* .

Identification of mutations in the flagella gene system

Using the same adhesion screening procedure and the same method to localize the Tn*10* in the chromosome of *E. coli* as described above, 12 different mini-Tn*10* insertions were found to be located in the flagellar gene system (see 32 for review). In Table 1, the nucleotide position of the transposon in the different flagella genes is indicated. Insertions were found in different genes of the class 2 *fliL* cluster at 40 min of the *E. coli* map. Four insertions were located in the *fliR* gene and one in the *fliP* gene. Both *fliR* and *fliP* encode putative components

of the type III flagellar export apparatus located in the flagellar basal body (13, 33). One insertion was found in *fliM*, encoding a 38 kDa protein, that has been suggested to be associated with the switch complex essential for flagella assembly, torque generation and direction of motor rotation (31, 34). Three mutations in the same region of the genome, in the class 2 *fliF* gene cluster were identified. Two of these insertions were located in the *fliG* gene whose product, FliG (37 kDa), is involved in flagellar assembly, torque generation and in the determination of the direction of flagella rotation (34). Additionally, one mutation was located in the *fliI* gene, encoding a 49 kDa flagellum specific ATPase which may play a role in the apparatus responsible for recognizing flagellar proteins to be transfered (12). Two other mutations were located in *flgA* and *flgG*, in region I at 23 min of the *E. coli* map, which contains many of the flagellar structural genes. The *flgG* gene, located in the *flgB* cluster, encodes a 28 kDa rod structural protein. The *flgA* product has not been identified in the structure, but may act as a chaperone in the P-ring assembly process (32). One insertion was found in the promoter region of the class 3 *fliC* gene at about 40 min. The *fliC* gene, encoding the flagellin protein FliC, is the last gene required in flagella morphological assembly (29).

Adhesion and growth of the flagella mutants

All of the flagella insertion mutants described above exhibited a reduced adhesion to polystyrene surface. Two types of adhesion profiles could be observed for these adhesion mutants. The first type contained all the flagella mutants except BGM11*fliC*, which composed the second type. The adhesion profiles of these groups of mutants are shown in Figure 3. The adhesion of the first group, composed of 11 different insertion mutants, is represented by BGM5 *fliR* strain. In all cases, the difference in binding between the parent strain and the mutants occurred early during incubation with the polystyrene surface. Adhesion of BGM5*fliR* was about 18% of the parent strain adhesion after 2 h, and increased slowly to reach a maximum of about 42% after 20 h. Strain BGM11 *fliC* showed better adhesion than the other group of mutants by 2 h of incubation. Attachment increased during 5 h and reached a maximum of about 82% after 13 h of contact. In the case of BGM11 *fliC*, differences in adhesion, when compared with the parent strain, were strongly reduced after 5 h of incubation. Furthermore, as observed for the parent strain, adhesion of the flagella mutants was reduced to the level of a *fim* mutant when D-mannose was added into the incubation medium (data not shown). The growth of the *fim* mutants was also affected when compared to the parent strain. The results shown in Figure 3 also indicate a direct correlation between adhesion and growth for the flagella mutants. The mutants that showed the poorest growth also exhibited the lowest

amount of adhesion.

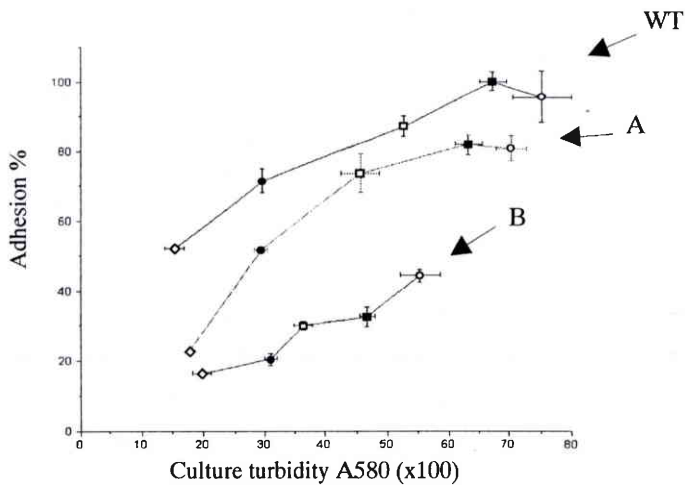

Fig. 3. Adhesion to polystyrene and growth of flagella mutants: WT, W3110 parent strain; A, BGM11 *fliC*; B, BGM5 *fliR*. Adhesion (%) and culture density (A_{580}) were measured at 2 h (◇), 5 h (●), 8.5 h (□), 13 h (■), and 20 h (○) after inoculation into the microplate. Adhesion is represented as percentage of adhesion compared to the wild type maximal adhesion. Error bars indicate the standard deviation based on triplicate measurements.

Motility and flagella morphology

To characterize the flagella morphology and motility properties of the flagella mutants, swarming plate assays plus phase-contrast and electron microscopy observations were performed. All the mutants, except BGM11 *fliC*, did not form halos after 6 h when stabbed into soft agar, and no motility was observed by phase-contrast microscopy analysis. Strains BGM11 *fliC* formed very tiny halos and showed a slow and disorganized movement under phase-contrast microscopy (Table 1). Transmission electron microscopy of the 12 flagella insertion mutants showed that the non-motile mutants did not produce flagella. Strain BGM14 *flgA* is shown as a typical example of a non flagellated isolated mutant in Figure 4B. Strain BGM11 *fliC* did produce short flagella in low numbers per cell (Fig. 4C). The flagella production of the parent strain was significantly higher, as expected (Fig. 4A).

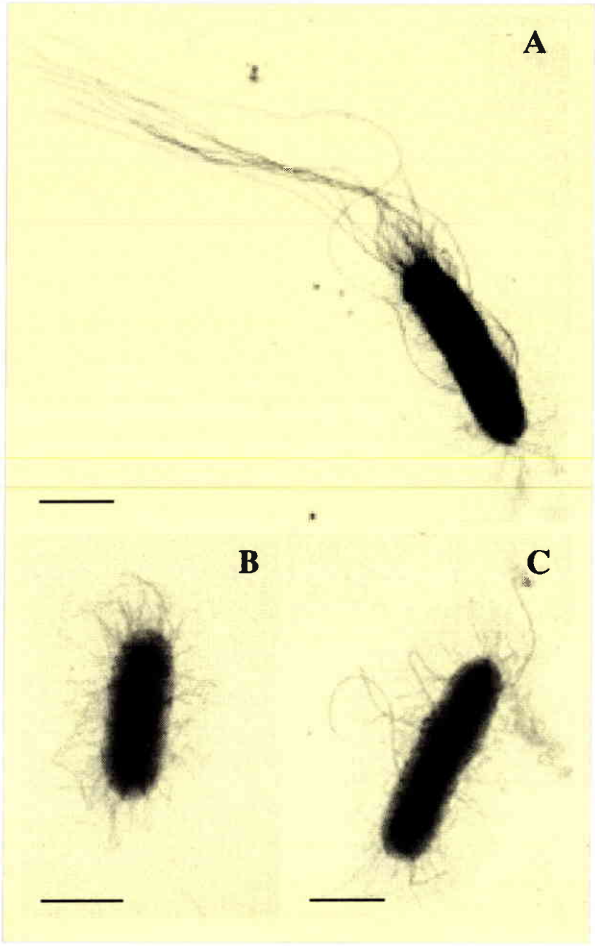

Fig. 4. Transmission electron micrographs analysis of negatively stained mutants and wild-type cells: Parent strain W3110 (A), BGF14 *flgA* (B), and BGM11 *fliC* (C). The size bar is equivalent to 1 μm.

Fimbriation in flagella mutated strains

To determine whether the altered adhesion properties of the flagella mutants was due to flagella alteration alone, or to a combined secondary effect related with a reduced type 1 fimbriae production in these mutants, haemagglutination tests with rabbit erythrocytes and spot blot analyses were carried out.

The haemagglutination assay showed different delays of time until agglutination occurred (Table 1). Haemagglutination was detected after about 30 sec for the parent strain, and after about 65 sec for the mutant BGM11 *fliC*. The group of flagella mutants, which showed the lowest adhesion (Fig. 3) was found to agglutinate red cells only after 150 to 180 seconds. The reduced ability to produce FimH specific binding with the mannose receptor, and therefore the

reduced amount of type 1 fimbriae, was confirmed by spot blot analysis (Fig. 5). The group of non-flagellated mutants with the lowest adhesion properties produced about 20% to 30% of type 1 fimbriae, whereas BGM11 *fliC*, the most adherent strain of the flagella mutants, produced 72% of type 1 fimbriae when compared to the parent strain.

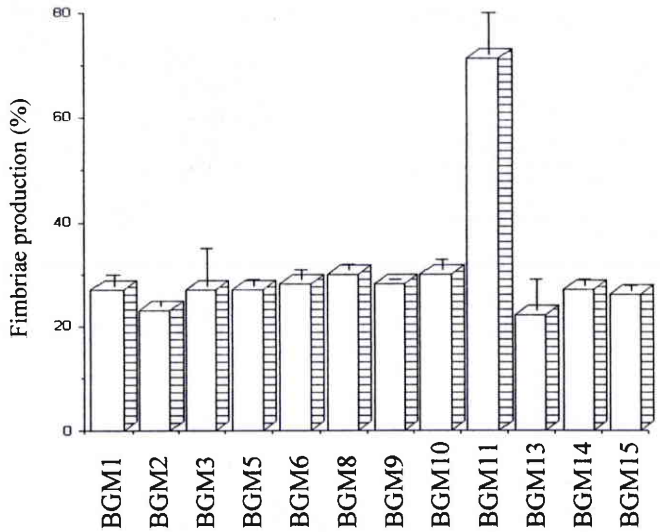

Fig. 5. Fimbriae type 1 production in flagella mutants. Values are represented as the percentage of fimbriae production compared to the parent strain. Error bars indicate the standard deviations based on duplicate measurements.

DISCUSSION

The interest in understanding polymer mediate attachment to non-biological surfaces led us to focus on the identification of genes associated with this phenomenon. A mini-Tn*10* random mutagenesis was carried out in the well-characterized *E. coli* K-12 strain W3110, and mutants with changes in binding properties to a relatively hydrophobic surface were isolated. Twenty-five chromosomal insertions leading to decreased attachment were located at different positions in the *fim* operon encoding for type 1 fimbriae.

The role of bacterial fimbriae in adhesion to abiotic surfaces has been previously observed (20, 39, 40). Rosenberg *et al.* (39) first reported that the thin fimbriae of *Acinetobacter calcoaceticus* significantly promoted adhesion to polystyrene and hydrocarbons. In contrast, fimbriated *Serratia marcescens* strains exhibited a lower adhesion to an air-water interface than the non-fimbriated ones (20). Furthermore, the presence of P pili was not

associated with the adhesive behavior of various urinary isolates of *E. coli* to polystyrene and urinary catheter material (silicone latex) (19, 37).

The mannose-sensitive type 1 fimbriae organelles (haemagglutination patterns inactivated by presence of D-mannose) were also associated with adhesion to abiotic surfaces. These organelles, are composed of about 1 000 copies of the major subunit protein FimA and of three minor ones, FimF, FimG, and FimH, which comprise about 1 % to 2 % of the total amount of fimbrial proteins (25). The adhesin FimH, associated with the two other minor subunits, is integrated at the tip and along the fimbrial rod. FimH was found to specifically bind mannose-oligosaccharide receptors on various cell surfaces (30).

Type 1 fimbriae of *Salmonella typhimurium* conferred a higher degree of adhesion to mineral particles such as albite, biotite, feldspar, magnetite and quartz (44). Jansen *et al.* (21) showed that adhesion to polyetherurethanes (synthetic polymers frequently used in medical devices) of *Klebsiella* strains possessing type 1 fimbriae was higher than for non-fimbriated variants. In this case, adding mannose to the medium inhibited adhesion, whereas no effect on adhesion was observed by adding other non-specific sugars. In *E. coli*, purified type 1 fimbriae were able to agglutinate polystyrene latex spheres (6). Significant correlation between the presence of mannose-sensitive fimbriae and early adhesion to polystyrene was observed for urinary isolates of *E. coli*. The authors observed that adhesion was inhibited when D-mannose was added to the medium (19).

In our case, (i) all the *fim* mutants exhibited very weak attachment independent of the time, the number of cells and the age of the culture, and did not express type 1 fimbriae, (ii) overproduction of fimbriae increased adherence, (iii) adhesion of the wild-type strain was reduced to the level of the *fim* mutants adhesion when D-mannose (the specific carbohydrate receptor of the FimH adhesin) was added to the incubation medium. These data indicate that, in addition to cell-host specific receptor recognition, type 1 fimbriae strongly perform adhesion to polystyrene surface. The mannose-sensitive adhesion we observed suggests that the specific adhesin FimH itself is a major determinant of synthetic polymer colonization.

In addition, the growth of the *fim* mutants was considerably affected. The cell factors regulating this response are unknown yet. However, the advantage of the fimbriated over the non-fimbriated bacteria in these growth conditions, is thought to be due to their ability to rapidly establish themselves in a pellicle on the surface of the broth, where their growth was promoted by the free supply of atmospheric oxygen (36).

Twelve mutations, in genes involved in flagella biosynthesis, leading to a decrease in adhesion were identified. Analyses of Motility, flagella morphology, growth, and type 1

fimbriae production, led us to discern two well-divided types of flagella-adhesion mutants. The majority of the flagella mutants (11 of 12) exhibited very reduced adhesion, slightly superior to the *fim* mutants, and were found to be non-motile, non-flagellated, and dramatically affected in the production of type 1 fimbriae. The mutant BGM11 *fliC* represented the second type of adhesion deficient mutant. This strain was found to be more adherent than the other group, to produce flagella (shorter and in a reduced amount per cell), to express a dysfunctional motility, and to express about 30% less fimbriae than the parent strain. The differences observed in flagella biosynthesis may be due to the positions of the Tn*10* insertion. The first group of mutants was affected in class 2 operons of the flagella gene system, and might have been considerably affected in the assembly pathway of the flagella apparatus (reviewed in 27). The insertion in BGM11 *fliC* was found in the promoter region of the class 3 *fliC* gene, encoding the flagellin subunits, required for the last step of flagella morphological assembly (27). In contrast to group of class 2 mutations, the *fliC* mutant may be able to produce the complete flagellar basal body, but synthesize a reduced amount of flagellin and therefore shorter flagella. As observed for the *fim* mutants, growth of the flagella mutants was affected when compared to the parent strain. The modifications in growth were correlated with the amount of type 1 fimbriae produced; the adhesion mutant with poorest growth exhibited the lowest amount of fimbriae. Considering that type 1 fimbriae considerably influence growth in static growth conditions (see above), the alterations in growth observed for the flagella mutants might have been mostly induced by the reduced fimbriae synthesis.

The effect of flagella mutations on type 1 fimbriae production is unknown. However, many virulence factors appear to share common regulatory features with the flagella system. In *Proteus mirabilis*, a mutant *flhA* (homologous to *E. coli* FlhA required for flagellar assembly) was non-motile and considerably affected in the expression of the virulence gene *hpmA* encoding haemolysin toxin (17). Arora *et al.* (1) identified two transcriptional regulators, FleQ and FleR, which regulated expression of both mucin adhesin and flagellar genes in *Pseudomonas aeruginosa*. In *Salmonella typhimurium*, mutations in the *flgM* flagella gene attenuated virulence in mice and exposed a clear link between the regulation of flagellar synthesis and other virulence factors (42). Furthermore, Ritter *et al.* (38) showed that disruption of the leucine-specific tRNA gene *leuX*, contained in the pathogenicity island PaiII of the uropathogenic *E. coli* strain 536 (O6:K15:H31), inactivated virulence factors such as haemolysin, Prf fimbriae and also flagella and type 1 fimbriae. The authors suggest that the important number of TTG leucine-specific codons present in the positive regulatory genes *fimB* for type 1 fimbriae and *flhC* for flagella might explain this common regulatory feature.

In this study, the amount of fimbriae was found to be correlated with the amount of flagella produced and with the motility exhibited on soft agar plates. Considering the fact that the mutation in *fliC* also affected fimbriae production, this effect may not be associated with an accumulation of flagellar structural proteins in the cell envelope that could disturb fimbriae export and assembly, but with cell regulated repression. The repression of fimbriae synthesis could be induced by an accumulation of flagella proteins in the cytoplasm, or by dysfunctional motility. The culture conditions used (static liquid medium), which favor growth of motile bacteria, may represent a hostile environment for non-motile bacteria. For example, the loss of motility induced by these mutations may mimic signals for a non-appropriated environment to colonize or for starvation conditions. The weak, but non-null motility exhibited by the *fliC* mutant could explain the differences in fimbriae expression observed between both, one of the *fim* regulatory genes, *fimB*, and of the major structural subunit gene, *fimA*, was repressed in stationary phase, probably indirectly, by the stationary phase-specific sigma factor RpoS. The negative control of regulatory factors such as RpoS on fimbriae production could be enhanced by loss of motility or by accumulation of flagellar proteins in the cytoplasm. At present, no data are available to identify this repressor(s), but it clearly appears that functional flagella are necessary for normal type 1 fimbriae expression.

Functional flagella have often been found to be associated with adhesion to surfaces. Korber *et al.* (28) showed that functional flagellum of *Pseudomonas fluorescens* permits the transport of bacteria from the aqueous phase to the boundary layer. In this case, the motile strain attached about four times more rapidly than the non-motile one, and achieved higher final adhesion amount. Tn*5* insertion mutants of *Pseudomonas fluorescens*, defective in the initial step of attachment to soil and seeds, have been found to be deficient in motility (9, 10). Furthermore, motility itself in *Vibrio fischeri* is required for successful light organ colonization (16). In our case, considering that the *fim* negative mutants were poorly adherent but fully flagellated and that the adhesion of wild-type and of the flagella mutants was reduced to the level of a *fim* mutant when D-mannose was added, the *E. coli* flagellum does not directly serves as polystyrene adhesin. However, the decreasing difference in adhesion versus time observed between the *filC* mutant and the wild type strain suggests that motility itself might have conferred advantages during polystyrene surface colonization. Furthermore, a strong correlation between the amount of type 1 fimbriae and the adhesion properties could be observed for the flagella mutants. Regarding the importance of type 1 fimbriae in adhesion to polystyrene, the impaired fimbriation might have strongly contributed to the loss of adhesion exhibited by these mutants.

In summary, this work further demonstrates the abilities of functional flagella and fimbriae to determine the adhesiveness of a given bacterium. The interaction found between these two cell surface organelles may reflect the adaptive potential of bacteria in response to environmental signals and prevent inappropriate colonization.

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REFERENCES

1. **Arora, S. K., B. W. Ritchings, E. C. Almira, S. Lory, and R. Ramphal.** 1997. A transcriptional activator, FleQ, regulates mucin adhesion and flagellar gene expression in *Pseudomonas aeruginosa* in a cascade manner. J. Bacteriol. **179:**5574-5581.

2. **Bachmann, B.J.** 1972. Pedigrees of some mutant strains of *Escherichia coli* K-12. Bacteriol. Rev. **36:** 525-557.

3. **Blomfield, I.C., M.S. Mc Clain , J.A. Princ , P.J. Calie, and B.I. Eisenstein.** 1991. Type 1 fimbriation and *fimE* mutants of *Escherichia coli* K-12. J. Bacteriol. **173:** 5298-5307.

4. **Boyd, A., and A. M. Chakrabarty.** 1995. *Pseudomonas aeruginosa* biofilms: role of the alginate exopolysaccharide. J. Indus. Microbiol. **15:**162-168.

5. **Boyer, H.W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli* . J. Mol. Biol. **41**: 459-472.

6. **Brinton, C. C., jr.** 1965. The structure, function, synthesis and genetic control of bacterial pili and molecular model for DNA and RNA transport in gram negative bacteria. Transactions of the New York Academy of Sciences. **27:**1003-1054.

7. **Costerton, J. W., K. –J. Cheng, G. G. Geesey, T. I. Ladd, J. C. Nickel, M. Dasgupta, and T. J. Marrie.** 1987. Bacterial biofilms in nature and disease. Ann. Rev. Microbiol. **41:**435-464.

8. **Costerton, J. W., Z. Lewandowski, D. E. Caldwell, D. R. Korber, and H. M. Lappin-Scott.** 1995. Microbial biofilms. Annu. Rev. Microbiol. **49:**711-745.

9. **DeFlaun, M. F., B. M. Marshall, E. P. Kulle, and S. B. Levy.** 1994. Tn*5* insertion mutants of *Pseudomonas fluorescens* defective in adhesion to soil and seeds. Appl. Environ. Microbiol. **60:**2637-2642.

10. **DeFlaun, M. F., A. Tanzer, A. L. McAteer, B. Marshall, and S. B. Levy.** 1990. Development of an adhesion assay and characterization of an adhesion-deficient mutant of *Pseudomonas fluorescens*. Appl. Environ. Microbiol. **56:**112-119.

11. **Dove, S. L., S. G. J. Smith, and C. J. Dorman.** 1997. Control of *Escherichia coli* type 1 fimbrial gene expression in stationary phase: a negative role for RpoS. Mol. Gen. Genet. **254:**13-20.

12. **Dreyfus, G., A. W. Williams, I. Kawagishi, and R. M. Macnab.** 1993. Genetic and biochemical analysis of *Salmonella typhimurium* FliI, a flagellar protein related to the catalytic subunit of the F_0F_1 ATPase and to virulence proteins of Mammalian and plant Pathogens. J. Bacteriol. **175:** 3131-3138.

13. **Fan, F., K. Ohnishi, N. R. Francis, and R. M. Macnab.** 1997. The FliP and FliR proteins of *Samonella typhimurium*, putative components of the type III flagellar export apparatus, are located in the flagellar basal body. Mol. Microbiol. **26:**1035-1046.

14. **Fletcher, M.** 1977. The effects of culture concentration and age, time, and temperature on bacterial attachment to polystyrene. Can. J. Microbiol. **23:** 1-6.

15. **Genevaux, P., S. Muller, and P. Bauda.** 1996. A rapid screening procedure to identify mini-Tn*10* insertion mutants of *Escherichia coli* K-12 with altered adhesion properties. FEMS Microbiol. Lett. **142:**27-30.

16. **Graf, J., P. V. Dunlap, and E. G. Ruby.** 1994. Effect of transposon-induced motility mutations on colonization of the host light organ by *Vibrio fischeri*. J. Bacteriol. **176:**6986-6991.

17. **Gygi, D., M. J. Bailey, C. Allison, and C. Hughes.** 1995. Requirement of FlhA in flagella assembly and swarm-cell differentiation by *Proteus mirabilis*. Mol. Microbiol. **15:**761-769.

18. **Hanson , M. S., and C.C. Brinton, jr.** 1988. Identification and characterization of the *Escherichia coli* type 1

pilus tip adhesion protein. Nature (London) **332:** 265-268.

19. **Harber, M. J., R. Mackenzie, and A. W. Asscher.** 1983. A rapid bioluminescence method for quantifying bacterial adhesion to polystyrene. J. Gen. Microbiol. **129:**621-632.

20. **Hermansson, M., S. Kjelleberg, T. K. Korhonen, and T. A. Stenström.** 1982. Hydrophobic and electrostatic characterization of surface structures of bacteria and its relationship to adhesion at an air-water surface. Arch. Microbiol. **131:**308-312.

21. **Jansen, B., J. Beuth, A. Pronzondo-Mordarska, S. Jansen, H. L. Ko, and G. Pulverer.** 1992. Adhesion of fimbriated and non-fimbriaeted *Klebsiella* strains to synthetic polymers. Zbl. Bakt. **276:**205-212.

22. **Kleckner, N., J. Blender, and J. Gottesman.** 1991. Uses of transposons with emphasis on Tn*10*. Methods Enzym. **204:** 139-180.

23. **Klemm, P.** 1986. Two regulatory *fim* genes, *fimB* and *fimE*, control the variation of type 1 fimbriae in *Escherichia coli*. EMBO J. **5:** 1389-1393.

24. **Klemm, P.** 1992. FimC, a chaperone like-periplasmic protein of *Escherichia coli* involved in biogenesis of type 1 fimbriae. Res. Microbiol. **143:** 831-840.

25. **Klemm, P, and K. A. Krogfelt.** 1994. Type 1 fimbriae of Escherichia coli, p. 9-26. In P. Klemm (ed.), Fimbriae: Adhesion, genetics, biogenesis. and vaccines. CRC Press, Ann Arbor, Mich.

26. **Klemm, P., and G. Christiansen.** 1990. The *fimD* gene required for cell surface localization of *Escherichia coli* type 1 fimbriae. Mol. Gen. Genet. **220:** 334-338.

27. **Klemm, P., B. J. Jorgensen, B. Kreft, and G. Christiansen.** 1995. The export system of type 1 and F1C fimbriae are interchangeable but work in parental pairs. J. Bacteriol. **177:** 621-627.

28. **Korber, D. R., J. R. Lawrence, and D. E. Caldwell.** 1994. Effect of motility on surface colonization and reproductive success of *Pseudomonas fluorescens* in dual-dilution continuous culture and batch culture systems. Appl. Environ. Microbiol. **60:**1421-1429.

29. **Kuwajima, G., J. I. Asaka, T. Fujiwara, K. Node, and E. Kondoh**. 1986. Nucleotide sequence of the *hag* gene encoding flagellin of *Escherichia coli*. J. Bacteriol. **168:** 1479-1483.

30. **Krogfelt, K. A., H. Bergmans, and P. Klemm.** 1990. Direct evidence that the FimH protein is the mannose specific adhesin of *Escherichia coli* type 1 fimbriae. Infect. Immun. **58:**1995-1998.

31. **Lloyd, S. A., H. Tang, X. Wang, S. Billings, and D. F. Blair.** 1996. Torque generation in the flagellar motor of *Escherichia coli*: Evidence of a direct role for FliG but not for FliM or FliN. J. Bacteriol. **178:** 223-231.

32. **Macnab, R.M.** 1996. Flagella and motility, p. 123-145. In F. C. Neidhart. *Escherichia coli* and *Salmonella typhimurium*: Cellular and molecular biology. ASM Press, Washington, D. C.

33. **Malakooti, J., B. Ely, and P. Matsumara.** 1994. Molecular characterization, nucleotide sequence, and expression of the *fliO*, *fliP*, *fliQ*, and *fliR* genes of *Escherichia coli*. J. Bacteriol. **176:** 189-197.

34. **Marykwas, D. L., and H. C. Berg.** 1996. A mutational analysis of interaction between FliG and FliM, two components of the flagellar motor of *Escherichia coli*. J. Bacteriol. **178:** 1289-1294.

35. **Miller, J.H.** 1992. A short course in bacterial genetics; A laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

36. **Old, D. C., and J. P. Duguid.** 1970. Selective outgrowth of fimbriate bacteria in static liquid medium. J. Bacteriol. **103:**447-456.

37. **Reid, G., H. C. van der Mei, C. Tleszer, and H. J. Busscher.** 1996. Uropathogenic *Escherichia coli* adhere to urinary catheters without using fimbriae. FEMS Immun. Med. Microbiol. **16:**159-162.

38. **Ritter, A., G. Blum, L. Emödy, M. Kerenyi, A. Böck, B. Neuhierl, W. Rabsch, F. Sceutz, and J. Hacker.** 1995. tRNA genes and pathogenicity islands: influence on virulence and metabolic properties of uropatogenic *Escherichia coli*. Mol. Microbiol. **17:**109-121.

39. **Rosenberg, M., E. A. Bayer, J. Delarea, and E. Rosenberg.** 1982. Role of thin fimbriae in adherence and growth of *Actinobacter caloaceticus* on hexadecane. Appl. Environ. Microbiol. **44:**929-937.

40. **Rosenberg, M., and S. Kjelleberg.** 1986. Hydrophobic interactions: Role in bacterial adhesion. In K. C. Marshall (ed). Advances in Microbial Ecology. **9:**353-393.

41. **Sambrook, J., E.F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

42. **Schmitt, C. K., S. C. Darnell, V. L. Tesh, B. A. D. Stocker, and A. D. O'Brien.** 1994. Mutation of *flgM* attenuates virulence of *Salmonella typhimurium*, and mutation in *fliA* represses the attenuated phenotype. J. Bacteriol. **176:**368-377.

43. **Shea, C., L. J. Lovelace, and H. E. Smith-Somerville.** 1995. *Deleya marina* as model organism for studies of bacterial colonization and biofilm formation. J. Indus. Microbiol. **15:**290-296.

44. **Stenström, T. A., and S. Kjelleberg.** 1985. Fimbriae mediated non-specific adhesion of *Salmonella typhimurium* to mineral particles. Arch. Microbiol. **143:**6-10.

45. **Weichenhan, D.** 1991. Fast recovery of DNA from agarose gels by centrifugation through blotting paper TIG. **7:**109

46. **Williams, V., and M. Fletcher.** 1996. *Pseudomonas fluorescens* adhesion and transport through porous media are affected by lipopolysaccharide composition. Appl. Environ. Microbiol. **62:**100-104.

Chapter IV

Identification of Tn*10* Insertions in the *rfaG*, *rfaP* and *galU* Genes Involved in Lipopolysaccharide Core Biosynthesis Affecting *Escherichia coli* K-12 Adhesion to Inanimate Surface

ABSTRACT

Tn*10* insertion mutants of *Escherichia coli* K-12 W3110 were selected for altered adhesion abilities to a polystyrene surface (P. Genevaux, S. Muller, and P. Bauda, FEMS Microbiol. Lett. 142:27-30, 1996). Seven insertion mutants that showed a decrease in adhesion harbored insertions in genes involved in lipopolysaccharide (LPS) core biosynthesis. Two insertions were located in the *rfaG* gene, two in the *rfaP* gene and three in the *galU* gene. These adhesion mutants were found to exhibit a deep rough phenotype and to be reduced, at different levels, in type 1 fimbriae production and motility. The loss of adhesion exhibited by these mutants was associated with either the affected type 1 fimbriae production and/or the dysfunctional motility. Apart from the pleiotropic effect of the mutations affecting LPS on type 1 fimbriae and flagella biosynthesis, no evidence for an involvement of the LPS itself in adhesion to polystyrene surface could be found.

INTRODUCTION

In most natural and artificial habitats, bacteria have a remarkable tendency to interact and grow in close association with surfaces (9, 10). Bacterial adhesion to biological and non-biological surfaces has been examined in many different fields, including human and animal diseases, industrial processes, and environmental microbiology (9). Adhesion to non-biological surfaces is a complex process in which bacteria utilize several mechanisms to adhere to various substrata under a plethora of environmental conditions. Some proteins and polysaccharides were found to influence adhesion to inanimate surfaces. Flagella and motility have often been associated with the initial step(s) of surface colonization (11, 12, 15, 16, 25). Experimental evidence indicated an important role of fimbriae in the early and late stages of the adhesion process (18, 14, 33, 34). Polysaccharide matrixes were found to be produced by attached bacteria (9). Exopolysaccharide in *Pseudomonas aeruginosa* and in *Delaya marina* were found to promote adhesion to surfaces under some environmental conditions (6, 39). In some cases, LPS was found to reduce attachment to relatively hydrophobic substrata (20, 45). To better understand which cell surface structures are required for a specific solid surface and how these adhesives interact with each other, a genetic approach using the well characterized bacterium *E. coli* K-12 and polystyrene as attachment surface was chosen.

We isolated mini-Tn*10* insertion mutants of *E. coli* K-12 with altered adhesion properties to a relatively hydrophobic surface, and localized the exact position of the transposon

in the mutated genes using inverse PCR and DNA sequencing. Here we describe transposon insertions located in the *rfaG*, *rfaP* and *galU* genes. These genes are involved in LPS biosynthesis of *E. coli* K-12 (reviewed in reference 40). These mutations specifically affect LPS inner core assembly and yield a deep rough phenotype that includes resistance to LPS-specific bacteriophages. Furthermore, these mutations show a decreased expression of some outer membrane proteins, as well as a increased susceptibility to hydrophobic compounds. In these adhesion mutants, the LPS alterations, the type 1 fimbriae production, the motility capability, and the induction of capsular polysaccharide were analyzed. No evidence for a direct involvement of the LPS itself in adhesion to a polystyrene surface could be found. The loss of adhesion was found mainly to be due to the pleitropic effect of the mutations on type 1 fimbriae and flagella biosynthesis, and possibly also of capsule expression.

MATERIALS AND METHODS

Bacterial strains, and growth conditions

A list of *E. coli* K-12 strains used in this study is provided in Table 1. The strains constructed for this work are mini-Tn*10* insertion mutants of *E. coli* K-12 W3110 (3) with altered adhesion properties (15). Cells were grown in static liquid LB media or LB agar plates (27) supplemented, when necessary, with 30 μg/ml of kanamycin. Resistance to novobiocin was determined by plating bacteria on LB supplemented with 10 μg/ml or 50 μg/ml of the antibiotic. Motility assays were carried out on soft agar plates containing 1% Tryptone, 0.5% NaCl, and 0.35% agar. Galactose fermentation assays were carried out in liquid MA minimal medium supplemented with 2% galactose as a carbon source (27). Sensitivity to bacteriophage P1*vir* was investigated as described by Miller (27).

DNA techniques, PCR methodology and nucleotide sequencing

All basic DNA procedures were carried out essentially as described by Sambrook *et al.* (36). Restriction endonucleases were used according to the manufacturer's specifications (New England Biolabs).

Localization of the mini-Tn*10* insertion mutations in the *E. coli* chromosome was carried out using inverse PCR. Two oligonucleotides annealing closely together at the IS*903* of the mini-Tn*10* (Km^r) derivative 103 (20) were prepared (primer I: 5'-TTA CAC TGA TGA ATG TTC CG-3', primer II: 5'-GTC AGC CTG AAT ACG CGT-3'). Chromosomal DNA of the

mutant strains was isolated and digested with either *Ava*II or *Hae*II. These restriction endonucleases did not digest the region between the 3' end of primer II and the end of the IS*10*. DNA restriction fragments were then circularized using T4 DNA ligase (Boehringer Mannheim). Subsequently, PCR using the circular DNA fragments was carried out with 50 pmol of each primer. The reactions were performed for 1 min at 96°C and then for 30 cycles each composed of 10 sec at 96°C, 30 sec at 55°C, and 2 min and 30 sec at 65°C. The PCR products were extracted from agarose gels by centrifugation through blotting paper (43) and purified by phenol/chloroform/isoamyl alcohol (50:48:2 by volume) extraction. Nucleotide sequencing was carried out using the Thermo Sequenase dye-terminator cycle sequencing kit from Amersham, with primer I as sequencing primer and double stranded PCR DNA fragments as template. Sequence analyses were carried out using the 373A semi-automated DNA sequencer of Applied Biosystems/Perkin-Elmer. DNA sequences were checked for homology in the Genbank library.

Table 1: Bacterial strains used in this study.

Strains	Revelant genotype[a]	Revelant phenotype			Source or reference
		Haemagglutination[b]	Novobiocin[c] A	B	
HB101	F⁻ *lacY1 recA13* Δ*fim*	-	NT	NT	(7)
W3110	F⁻ λ⁻ IN(*rrnD-rrnE*)1 *rph*-1	31	+	+	(1)
BGM5	*fliR*	NT	NT	NT	(14)
BGR1	*rfaG* 1638	48	-	-	this study
BGR2	*rfaG* 2095	48	-	-	"
BGR3	*rfaP* 2698	> 60	-	-	"
BGR4	*rfaP* 2730	> 60	-	-	"
BGU1	*galU* 154	35	+	+	"
BGU2	*galU* 488	> 60	+	-	"
BGU3	*galU* 697	> 60	+	-	"

[a] The name of the mutations obtained in this work corresponds to the nucleotide positions of the mini-Tn*10* in the DNA nucleotide sequence corresponding to the following Genbank accession numbers; M80599 for the *rfaP* and *rfaG* and M98830 for the *galU* insertions.
[b] Time in seconds until agglutination occurred. Average value of six measurements
[c] Growth on LB plates supplemented with 10 μg/ml (A) and 50 μg/ml (B) of novobiocin.

Adhesion experiments

Adhesion experiments were carried out using polystyrene microtiter plates essentially as described previously (15). Briefly, the cells were grown to an A_{580}=0.70 in LB medium.

Aliquots of 20 µl of the cell cultures were pipetted into the wells of a polystyrene microtiter plate containing 200 µl of fresh LB (+Km) medium. Cells were then allowed to grow and adhere for 2 h, 5h, 8.5h and 20h at 37°C. At the end of that period, the culture turbidity in the wells were measured (A_{580}) and unbound cells were removed. Adhered cells were fixed for 30 min at 80°C and stained with crystal violet (0.1%). Decolouring solution (ethanol/acetone, 80%: 20%) was added and the eluted stain was estimated photometrically at 580 nm.

Type 1 fimbriae production

The capacity of bacteria to produce type 1 fimbriae was determined by spot blot analysis. Single colonies of each strain were grown at 37°C in static LB medium to an A_{580}=0.70. Cells were then collected by centrifugation (30 sec at 7 000 x *g*), and resuspended in phosphate-buffered saline (136.9 mM NaCl, 2.7 mM KCl, 10.0 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7.2). Serial dilutions of the cell suspensions were spotted onto a 0.2 µm nitrocellulose membrane paper, using the Bio-Dot apparatus from Bio-Rad. The membrane papers were then first incubated with polyclonal rabbit antibodies directed against type 1 fimbriae, kindly provided by Maryvonne Dho-Moulin (INRA. Tours, France). The blots were then incubated with goat anti-Rabbit conjugated to horse radish peroxidase as secondary antibodies. Detection was carried out using the ECL kit from Amersham. Spots were analyzed with the Nih-image program version 1.56.

The capacity of bacterial strains to express a D-mannose-binding phenotype was assayed by their ability to agglutinate rabbit erythrocytes. Aliquots of cells in 50 µl of phosphate-buffered saline (A_{580}=10.0) were mixed with 50 µl of 5% erythrocytes in KRT solution (128.3 mM NaCl, 5.1 mM KCl, 1.3 mM $MgSO_4.7H_2O$, 2.7 mM $CaCl_2.2H_2O$, 10.0 mM Tris-HCl, pH 7.4) at room temperature on glass slides, and the period until agglutination occurred was determined and taken as measure for agglutination.

Outer membrane preparation

Cells were grown to an A_{580}=0.7 at 37°C, washed with phosphate-buffered saline, and concentrated to A_{580}=2.0 in a solution containing 50 mM Tris and 2 mM EDTA (pH 8.5). Samples were then frozen and thawed three times and sonicated three times for 10 sec. Cell debris were collected by centrifugation (30 min at 13000 x *g*, 4°C) and the pellet fraction,

containing outer membranes, was resuspended in 100 ml of phosphate-buffered saline. Proteinase K (1 mg/ml) was added and samples were incubated for 1 h at 60 °C.

Gel electrophoretic analysis and silver staining of LPS

For analysis of LPS, the outer membrane preparations were analyzed by gel electrophoresis using 18% polyacrylamide gels and a (SDS)-tricine buffer system (37). Silver staining of the LPS was carried out as described by Tsai and Frasch (41).

RESULTS

Isolation of *rfaG*, *rfaP* and *galU* mutants

A total of 7 000 *E. coli* K-12 W3110::mini-Tn*10* insertion mutants were screened for their ability to adhere to polystyrene microtiter plates (15). Seven mutants that expressed a reduced adhesion harbored the mini-Tn*10* in genes involved in LPS core biosynthesis (Table 1). The precise position of the mini-Tn*10* in the affected gene/gene cluster in the *E. coli* chromosome was carried out using inverse PCR and nucleotide sequence analysis. Four mutants had an insertion in the *rfa* gene cluster, at about 81 min of the *E. coli* chromosome (40). Two of these *rfa* mutants harbored the mutation at different positions in the*rfaG* gene, whose product is involved in the transfer of glucose from UDP-glucose to heptose II in the inner core of the LPS structure (30). The two other insertions in the *rfa* cluster were located in the *rfaP* gene, associated with two distinct inner-core functions: the attachment of phosphoryl substituent to heptose I, and the attachment of a branch heptose III to the heptose II residue (30).

Three other mutants harbored the mutation in the *galU* gene, encoding UTP:α-D-glucose-1-phosphate uridylyltransferase necessary for the synthesis of UDP-glucose, a central element in the synthesis of components of the cell envelope of*E. coli*, at about 24 min of the *E. coli* chromosome (38, 44). In strains BGU2 and BGU3, the transposon was located in the structural gene, whereas in strain BGU1, the transposon insertion was found in the upstream region (position -80 bp) of the *galU* structural gene (Table 1).

LPS lesions

To examine the lesions of the LPS in the *rfaG*, *rfaP* and *galU* mutants, outer membrane extracts treated with proteinase K were analyzed by tricine-(SDS) polyacrylamide gel electrophoresis. Figure 1 represents a comparison of the LPS gel profile of the parent strain

W3110 and those of the Tn*10* insertions mutants.

The *rfaG* and the *rfaP* mutants had a fast migrating truncated LPS, as compared with the parent LPS. The *rfaG* insertion mutants produced an identical and rapidly migrating LPS, whereas the two *rfaP* mutants produced a slower migrating LPS. These results are in accordance with previous studies of *E. coli* LPS (2). The *rfaG*::Tn*10* mutants exhibit a chemotype Rd1 due to the lack of glucose on Heptose II and Heptose III in the inner core region of the LPS (2). The Tn*10* insertions in *rfaP* produced chemotype Rc, including the first glucose residue on the Heptose II (2).

The *galU* mutants showed two different patterns of LPS migration. The strains BGU2 and BGU3, in which the transposon is located in the *galU* structural gene, exhibited an identical truncated LPS, which migrated close to the LPS of the *rfaG* mutants. This is in agreement with the fact that *galU* mutants, which are unable to provide UDP-glucose, the substrate for *rfaG*, produce an incomplete lipopolysaccharide lacking all the sugars beyond the heptose residues (32, 41). Mutant BGU1 produced two bands of LPS, one migrating like the parent strain LPS, and one like the two other *galU* mutants. The leaky phenotype exhibited by the insertion at position -80 bp of the *galU* gene might be due to impaired expression of the *galU* gene. In addition, *galU* expression is essential for growth on galactose. Growth analyses in minimal media supplemented with galactose as the sole carbon source were carried out. The parent strain had a growth rate of 0.28 h^{-1}, the mutant BGU1 of about 0.17 h^{-1}, whereas the mutants BGU2 and BGU3 were not able to grow. These results confirmed that BGU1 was still able to produce an active GalU.

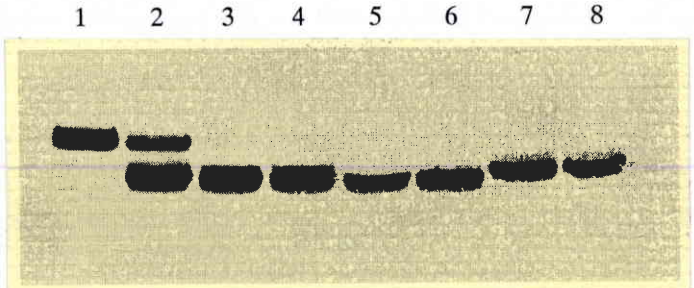

Fig. 1. Silver-stained gel of LPS from the wild type strain and the adhesion mutants. Lanes: 1, LPS from the parent W3110; 2, BGU1 *galU*; 3 and 4, BGU2 *galU* and BGU3 *galU*; 5 and 6, BGR1 *rfaG* and BGR2 *rfaG*; 7 and 8, BGR3 *rfaP* and BGR4 *rfaP*.

Sensitivity to novobiocin and colanic acid production

Sensitivity to hydrophobic antibiotics, such as novobiocin, is a characteristic of mutants with a deep rough phenotype (30). To determine this phenotype in the isolated adhesion mutants, a growth assay on LB plates supplemented with novobiocin (10 μg/ml and 50 μg/ml) was carried out. As shown in Table 1, the strains mutated in the *rfa* cluster could not grow at both concentrations, whereas the growth of the parent strain was not affected. These results are in agreement with Parker *et al.* (30) and confirmed the deep rough character of the isolated *rfa* mutations.

The *galU* insertion mutants were also checked for resistance to novobiocin (Table 1). These mutants grew as well as the parent strain when plated on LB supplemented with 10μg/ml of the antibiotic. Growth with 50 μg/ml of novobiocin was inhibited for strains BGU2 and BGU3. These *galU* mutants may exhibit a partial deep rough phenotype. No sensitivity to novobiocin was observed for BGU1.

Deep rough mutations can induce the production of colanic acid capsular polysaccharide (30). Mucoid colonies on agar plates and resistance to bacteriophage P1 are observed when colanic acid is produced (30). In our case, only the two *rfaP* insertion mutants exhibited mucoid colonies and were resistant to P1. In contrast, the *rfaG* and the *galU* mutants showed no mucoid colonies and were sensitive to P1 when compared with the parent strain.

Motility and fimbriation

The deep rough mutants produce a reduced amount of some outer membrane proteins (1, 24). To determine the effect of the *rfaG*, *rfaP* or *galU* mutations on structures potentially involved in adhesion, motility and type 1 fimbriae production were analyzed.

To evaluate the motility capability of the adhesion mutants, swarming plate assays were carried out (Fig. 2). The non-motile flagella mutant BGM5*fliR* (14) served as a negative control and motility was measured 6 h after incubation. The mutants BGR1 and BGR2 (*rfaG*) were still able to form growth zones of approximately half size of those of the parent strain W3110. The *rfaP* mutants BGR3 and BGR4 produced smaller zones than the *rfaG* strains. The strain BGU1 *galU* was also considerably affected in motility. Mutants BGU2 and BGU3 produced very tiny halos, almost undetectable after 6 h of incubation. These results showed that the LPS mutations resulted in different degrees of reduced motility.

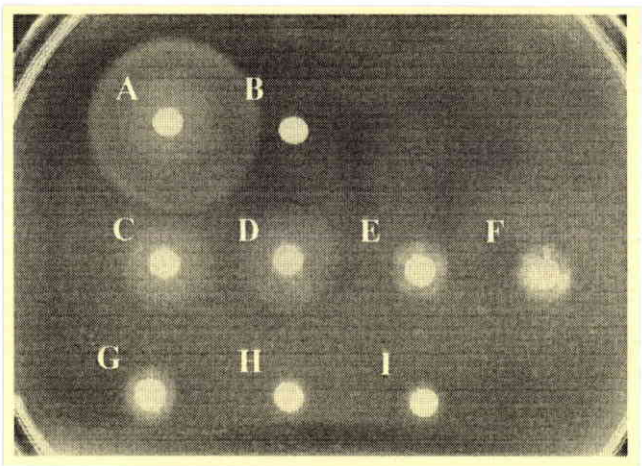

Fig. 2. Motility of mutants and wild-type strain on soft agar plates after 6 h at 37°C: A, parent strain W3110; B, BGM5 *fliR*; C and D, BGR1 and BGR2 (*rfaG*); E and F, BGR3 and BGR4 (*rfaP*); G, BGU1 (*galU*); H and I, BGU2 and BGU3 (*galU*).

In order to evaluate type 1 fimbriae production in the *rfa*G, *rfaP* and *galU* mutants, spot blot analyses using anti-type 1 fimbriae polyclonal antibodies were carried out (Fig. 3). The non-fimbriated strain HB101 Δ*fim* (7) was used as negative control (data not shown). The *rfaG* mutants BGR1 and BGR2 were found to express about 50% of the fimbriae, and the *rfaP* BGR3 and BGR4 about 30% when compared to the parent strain. No significant differences were observed between the two *galU* mutants BGU2 and BGU3 and the *rfaP* mutants. However, the *galU* mutant that exhibited a leaky phenotype (see above) showed no significant decrease in fimbriae production.

Type 1 fimbriae produce an adhesin, FimH, that binds specifically to the mannose receptor at the surface of various red blood cells (see 22 for a review). The ability of the *rfa* and *galU* mutants to agglutinate rabbit erythrocytes, and therefore to produce adhesive type 1 fimbriae, was assayed by measuring the time needed for agglutination to occur (Table 1). No significant differences were observed in the *rfaP* and the *galU* mutants, which nonetheless showed haemagglutination at about 30 sec later than the parent strain, while *rfaG* mutants agglutinated red cells 15 seconds after W3110. No significant differences were observed between BGU1 and the parent strain.

The results obtained by the spot blot analyses and the haemagglutination assays showed different levels of alterations of the type 1 fimbriae in these mutants.

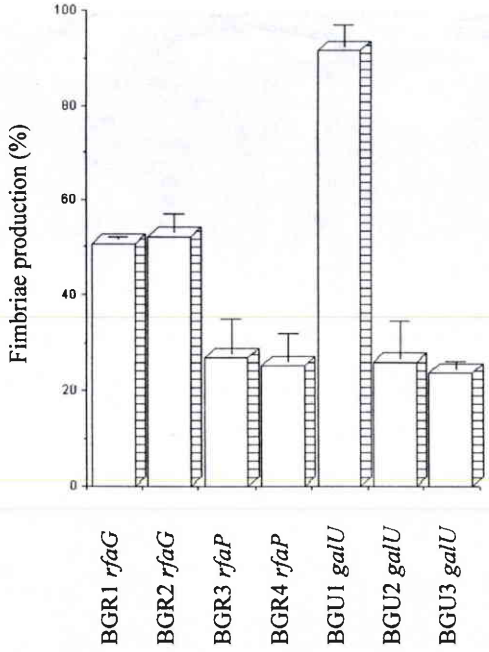

Fig. 3. Type 1 fimbriae production of the *rfa* and *galU* mutants by spot blot analyses. Values are represented as percentage of fimbriae production compared to the parent strain. Error bars indicate the standard deviation based on duplicate measurements.

Adhesion properties

The *rfaG*, *rfaP* and *galU* insertion mutants were analyzed for adhesion to polystyrene microtiter plates. Adhesion and culture turbidity in the wells were measured at various times after inoculation. Figure 4 shows the adhesion abilities of the parent strain and of the mutants obtained.

The parent strain's adhesion increased linearly for up to 13 h. Maximal adhesion was obtained at this time, while a slight decrease was observed after 20 h of inoculation.

Out of the seven mutations identified, three groups of adherents could be distinguished: The first contained BGU1 *galU*, the second group contained the two *rfaG* mutants, and the third group contained the two *rfaP* and two *galU* mutants BGU2 and BGU3. After 2 h of incubation, the *rfaG* mutants adhered about 10% less than strain W3110, while the mutants of the third group showed 37% less adhesion. However, both mutants produced a maximal adhesion versus time, higher than strain W3110 between 2 h and 5 h. The *rfaG* mutants reached the number of adhered cells of the parent after 5 h while the *rfaP* and the two *galU* mutants BGU2 and BGU3 showed an adhesion 35% inferior to strain W3110. A consequent decrease in adherent cells versus time was observed for both mutations after that period of time. A final plateau of about 78% of adhesion compared with strain W3110 for the *rfaG* mutants and 52% for the *rfaP* and

the two *galU* mutants BGU2 and BGU3 was reached after 8.5 h. As noticed for W3110, a slight decrease in the percentage of adhesion was also observed for both strains after 20 h. Strain BGU1, which expressed a leaky phenotype, showed the same percentage of adhesion as the *rfaG* mutants after 2 hours and 10% less after 5 h. The adhesion between 8.5 h and 13 h was very similar to the level of the parent but a significant decrease was observed after 20 h.

In addition, when 2.5% D-mannose, the specific receptor for the type 1 fimbriae adhesin, was added into the incubation medium during the assay, the adhesion of the parent strain and of the LPS mutants were identical and could only reach a maximal level of about 25% of that observed in the absence of D-mannose (data not shown).

Besides adhesion, changes in growth were observed. The mutants with the poorest growth exhibited the lowest final adhesion (see Discussion).

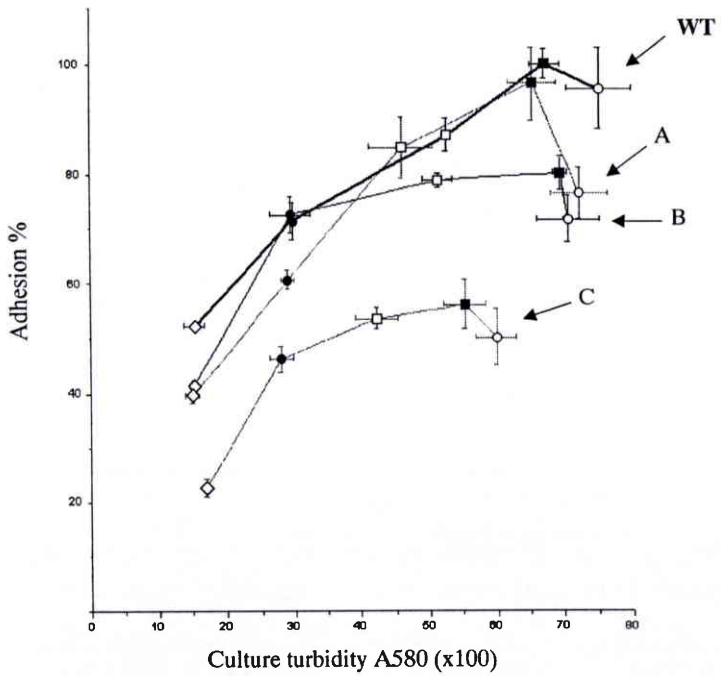

Fig. 4. Adhesion and growth properties of mutants and wild-type strain: WT, parent strain W3110; A, BGU1 (*galU*); B, BGR1 and BGR2 (*rfaG*); C, BGR3 and BGR4 (*rfaP*), and BGU2 and BGU3 (*galU*). Adhesion (%) and culture density (A_{580}) were measured 2 hours (□) 5 hours (●), 8.5 hours (▫), 13 hours (■) and 20 hours (○) after inoculation. Adhesion is represented as percentage of adhesion compared to the wild type maximal adhesion. Error bars indicate the standard deviation based on triplicate measurements.

DISCUSSION

Adhesion to biological and non-biological surfaces is a major characteristic of bacteria (9, 26). Mutagenesis has been found to be a powerful approach to identify bacterial surface components involved in specific adhesive interactions with biological surfaces (19, 22). However, perhaps due to a lack of specific receptors, adhesion to abiotic surfaces has been more difficult to address. The interest in understanding polymers that mediate attachment to inanimate surfaces led us to focus on the identification of genes associated with this phenomenon. We randomly mutated genes of *E. coli* K-12 using mini-Tn*10* transposon mutagenesis, and selected mutants with changes in binding properties to a polystyrene surface. Seven of these adhesion mutants harbored the mutation in genes involved in LPS synthesis. Two inserts were found in the *rfaG* gene, and two in *the rfaP* gene, both genes involved in LPS core biosynthesis. Three other insertions were found in the *galU* gene which provide UDP-glucose to RfaG, necessary for LPS biosynthesis (2, 44). The *rfaG* and *rfaP* mutants isolated, as well as the two *galU* null mutants BGU2 and BGU3, exhibited a deep rough phenotype. The deep rough phenotype is a complex phenotype exhibited by a subset of LPS mutations. This phenotype was found to be induced by the lack of the phosphate-containing substituents on heptose, or by a defect in the heptose region of the inner-core. This phenotype includes alterations in LPS, sensitivity to detergents and hydrophobic antibiotics such as novobiocin, elevation of the phosphatidylethanolamine content of the outer membrane, reduction in some outer membrane proteins, resistance to various bacteriophages, and an increase in outer membrane phospholipids (32, 40). The *rfaP* gene product was found to be directly involved in the deep rough phenotype, whereas the deep rough phenotype exhibited by some *rfaG* mutants might be due to strong polarity of the mutation on adjacent genes such as *rfaP* (30). The partially deep rough phenotype exhibited by the *galU* mutants may be due to the decrease in the *tolC* gene product in these mutants, which is involved in the conversion of phosphoryllethanolamine (PPEA) to free phosphate on heptose I after export of the LPS to the outer membrane (40, 42).

Cell surfaces analysis demonstrated important lesions in the LPS structure for all the mutants. All these adhesion mutants showed a general decrease in attachment to polystyrene. Williams *et al.* (45) recently demonstrated the importance of LPS composition in the adhesion of *Pseudomonas fluorescens* to surfaces of various levels of hydrophobicity. Changes in binding properties were observed for strains with attenuated or with a complete lack of the O antigen in the LPS structure. In contrast with our results obtained with *E. coli* K-12, which has no O

antigen, an increase in adhesion to polystyrene was observed. In *Salmonella typhimurium*, the presence of intact LPS was found to reduce cell surface hydrophobicity and therefore to reduce adhesion to relatively hydrophobic substrata (18). The lack of LPS might expose the lipid moiety in the LPS mutants and in this way increase cell surface hydrophobicity (45).

In our case, in spite of the very similar LPS lesions produced in the two *rfaG* mutants and in the BGU2 and BGU3 *galU* mutants, important differences in adhesion were observed. Moreover, the *rfaP* adhesion mutants, which produced a less truncated LPS than the other mutants, showed the same adhesion as the strains BGU2 and BGU3 *galU*. These observations did not permit conclusions about a direct effect of *E. coli* K-12 LPS in adhesion to a polystyrene surface. In order to evaluate the production of surface organelles that may play a role in adhesion in these deep rough mutants, type 1 fimbriae production and motility capability were determined.

Type 1 fimbriae and motility were affected to different extents by the *rfa* and *galU* mutations. The effect of deep rough mutations on fimbriae production was previously observed by Philipicinec *et al.* (31) that showed that an *rfaQ*::Tn*10* insertion affected production of K99 fimbriae probably because of a reduced incorporation of the usher in the outer membrane and/or a hampered transport of the major subunits across the outer membrane. LPS deep rough mutations have also been associated with a loss of flagella expression. Parker *et al.* (30) observed a lack of flagella in a *rfaGPBI* deletion mutant of *E. coli* K-12 (30). An early study showed that *galU* mutants produced a very small number of flagellar filaments and hooks (23). The manner in which LPS interacts with outer membrane structures is not clear. Recently Missiakas *et al.* (28) showed that a *rfaD* mutation that produced a deep rough phenotype increased expression of the sigma E regulon, which is also specifically induced by misfolded proteins in the periplasm. In this mutant, the wild-type-like protein content of the outer membrane, in spite of the defect in LPS, was restored by overexpression of the extracytoplasmic chaperone SurA (28). Such a defective LPS may be unable to participate in the biosynthesis of outer membrane proteins, which then results in an augmented protein misfolding and degradation.

Bacterial fimbriae have been associated with adhesion to inanimate surfaces (18, 34, 33). Surface components such as fimbriae are known to play a role in the initial step of adhesion, but often serve to more firmly anchor the bacteria (20). Type 1 fimbriae have been found to strongly promote adhesion to polystyrene in *E. coli* K-12 independently of the number of cells, of the age of the culture, and of the presence of flagella (17, 14).

In our case, type 1 fimbriae analysis pointed to a correlation between the presence of

fimbriae and adhesion to polystyrene. Furthermore, addition of D-mannose, the specific carbohydrate receptor of the type 1 fimbriae adhesin, reduced the adhesion of the parent strain and of the LPS mutants to the same low level. These results suggest that the pleitropic effect of the LPS mutations on type 1 fimbriae biosynthesis was the main cause of loss of adhesion to polystyrene.

The decrease in adherent cells versus time observed for the parent strain and for the BGU1 *galU* mutant after 13 h, and earlier for the other mutants, may be due in large measure to changes in fimbriae expression. Recently, Dove *et al.* (13) showed that type 1 fimbriae expression was repressed in early stationary phase by the RpoS stationary phase specific-sigma factor. In addition, the superior adhesion versus time, when compared to the wild-type strain, observed with the *rfaG* mutants after 5 hours of inoculation, might be due to growth-phase-dependent expression of fimbriae recently observed with haemolysin secretion (4). Bauer *et al.* (4) showed that a deep rough *rfaC* mutant was affected for haemolysin secretion and activity in a growth-phase-dependent manner. In contrast to the wild-type strain, hemolytic activity in this mutant peaked during mid-exponential phase of growth and was rapidly and dramatically reduced in the late-exponential phase of growth. In our case, the important increase in adhesion versus time exhibited by the *rfaG* mutants after 5 h of inoculation might be explained by growth-phase-variations in type 1 fimbriae production as observed for the secreted hemolysin (4).

Growth of the LPS mutants appeared to be affected in an adhesion-correlated manner. Weaker adherents produced less type 1 fimbriae and exhibited the poorest growth. The alterations in growth may be due to the reduced amount of type 1 fimbriae produced by these mutants. The presence of type 1 fimbriae was found to considerably favor growth in static liquid growth conditions (8, 29).

Bacterial flagella have often been associated with the initial step of adhesion to surfaces (11, 12, 16, 25). Korber *et al.* (25) showed that functional flagella of *Pseudomonas fluorescens* permitted the transport of cells from the bulk aqueous phase to the boundary layer. The motile strain attached about four times more rapidly and achieved a higher final adhesion level than the non-motile one. Motility itself was required for successful light organ colonization by *Vibrio fischeri* (16). Furthermore, Tn*5* insertion mutants of *Pseudomonas fluorescens*, defective in the initial stage of attachment to soil and seeds, have been found to be deficient in motility (11, 12). A recent study of *E. coli* K-12 adhesion to polystyrene showed that motility provided by flagella, but not the flagellum itself, conferred advantages during surface colonization (14). In our case, the loss of motility exhibited by the adhesion mutants may have influenced the

adhesion pattern. This could explain the important differences in adhesion obtained between the strain BGU1 *galU* and the parent strain W3110, and between the *rfaG* mutants and the parent strain W3110, after 2 h of inoculation.

The two *rfaP* adhesion mutants were found to express colanic acid capsular polysaccharide. The presence of capsule in enterotoxigenic *E. coli* strains was found to decrease adhesion to epithelial cells of pigs. The observed loss of adhesion was not due directly to the presence of the capsular material but to an impeded fimbriae-mediated adhesion induced by the capsule (35). In our case, capsule induction in the *rfaP* mutants could have impeded the type 1 fimbriae-mediated adhesion to polystyrene and could explain the differences in type 1 fimbriae production measured between the two *rfaG* and the two *rfaP* mutants. Furthermore, capsule induction could have also disturbed flagellar movement and therefore could explain the differences in motility observed between the *rfaG* and the *rfaP* mutants. Further investigations are required to confirm this hypothesis.

In conclusion, this study further demonstrates the pleitropic character of mutations affecting LPS biosynthesis, and the major role of type 1 fimbriae in adhesion to polystyrene. Furthermore, apart from the indirect effect of the LPS mutations on the production of type 1 fimbriae and flagella, no evidences for a direct involvement of the *E. coli* K-12 LPS in adhesion to polystyrene could be found.

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REFERENCES

1. **Ames, G. F. L., E. N. Spudich, and H. Nikaldo.** 1974. Protein composition of the outer membrane of *Salmonella typhimurium*: effect of lipopolysaccharide mutations. J. Bacteriol. **117:**406-416.2.

2. **Austin, E. A., J.F. Graves, L. A. Hite, C. T. Parker, and C. A. Schnaitman.** 1990. Genetic analysis of lipopolysaccharide core biosynthesis by *Escherichia coli* K-12: Insertion mutagenesis of the *rfa* locus. J. Bacteriol. **172:**5312-5325.

3. **Bachmann, B.J.** 1972. Pedigrees of some mutant strains of *Escherichia coli* K-12. Bacteriol. Rev. **36:** 525-557.

4. **Bauer, M. E., and R. A. Welch.** 1997. Pleiotropic effects of a mutation in *rfaC* on *Escherichia coli* hemolysin. Infect. Immun. **65:**2218-2224.

5. **Blomfield I.C., M.S. Mc Clain, J.A. Princ, P.J. Calie, and B.I. Eisenstein.** 1991. Type 1 fimbriation and *fimE* mutants of *Escherichia coli* K-12. J. Bacteriol. **173:**5298-5307.

6. **Boyd, A., and A. M. Chakrabarty.** 1995. *Pseudomonas aeruginosa* biofilms: role of the alginate exopolysaccharide. J. Indus. Microbiol. **15:**162-168.

7. **Boyer, H.W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**:459-472.

8. **Brinton, C. C., jr.** 1965. The structure, function, synthesis and genetic control of bacterial pili and molecular model for DNA and RNA transport in gram negative bacteria. Transactions of the New York Academy of Sciences. **27:**1003-1054.

9. **Costerton, J. W., K. –J. Cheng, G. G. Geesey, T. I. Ladd, J. C. Nickel, M. Dasgupta, and T. J. Marrie.** 1987. Bacterial biofilms in nature and disease. Ann. Rev. Microbiol. **41:**435-464.

10. **Costerton, J. W., Z. Lewandowski, D. E. Caldwell, D. R. Korber, and H. M. Lappin-Scott.** 1995. Microbial biofilms. Annu. Rev. Microbiol. **49:**711-745.

11. **DeFlaun, M. F., B. M. Marshall, E. P. Kulle, and S. B. Levy.** 1994. Tn*5* insertion mutants of *Pseudomonas fluorescens* defective in adhesion to soil and seeds. Appl. Environ. Microbiol. **60:**2637-2642.

12. **DeFlaun, M. F., A. Tanzer, A. L. McAteer, B. Marshall, and S. B. Levy.** 1990. Development of an adhesion assay and characterization of an adhesion-deficient mutant of *Pseudomonas fluorescens*. Appl. Environ. Microbiol. **56:**112-119.

13. **Dove, S. L., S. G. J. Smith, and C. J. Dorman.** 1997. Control of *Escherichia coli* type 1 fimbrial gene expression in stationary phase: a negative role for RpoS. Mol. Gen. Genet. **254:**13-20.

14. **Genevaux, P., M. S. DuBow, B. Oudega, and P. Bauda.** The role of flagella and type 1 fimbriae in *Escherichia coli* K-12 adhesion to inanimate surface. *Chapter III*.

15. **Genevaux, P., S. Muller, and P. Bauda.** 1996. A rapid screening procedure to identify mini-Tn*10* insertion mutants of *Escherichia coli* K-12 with altered adhesion properties. FEMS Microbiol. Lett. **142:**27-30.

16. **Graf, J., P. V. Dunlap, and E. G. Ruby.** 1994. Effect of transposon-induced motility mutations on colonization of the host light organ by *Vibrio fischeri*. J. Bacteriol. **176:**6986-6991.

17. **Harber, M. J., R. Mackenzie, and A. W. Asscher.** 1983. A rapid bioluminescence method for quantifying bacterial adhesion to polystyrene. J. Gen. Microbiol. **129:**621-632.

18. **Hermansson, M., S. Kjelleberg, T. K. Korhonen, and T. A. Stenström.** 1982. Hydrophobic and electrostatic characterization of surface structures of bacteria and its relationship to adhesion at an air-water surface. Arch. Microbiol. **131:**308-312.

19. **Kado, C. I., and J. H. Crosa** (ed). 1994. Molecular mechanisms of bacterial virulence. Kluwer Academic Publishers, Dodrecht.

20. **Kjelleberg, S.** 1984. Adhesion to inanimate surfaces. In K. C. Marshall (ed). Microbial adhesion and aggregation. Dahlem Konferenzen. Springer, Berlin.

21. **Kleckner, N., J. Blender, and J. Gottesman.** 1991. Uses of transposons with emphasis on Tn*10*. Meth. Enzym. **204:**139-180.

22. **Klemm, P., and K. A. Krogfelt.** 1994. Type 1 fimbriae of *Escherichia coli*, p. 9-26. In P. Klemm (ed.), Fimbriae: Adhesion, genetics, biogenesis. and vaccines. CRC Press, Ann Arbor, Mich.

23. **Komeda, Y., T. Icho, and T. Iino.** 1977. Effects of *galU* mutation on flagellar formation in *Escherichia coli*. J. Bacteriol. **129:**908-915.

24. **Koplow, J., and H. Goldfine.** 1974. Alterations in the outer membrane of the cell envelope of heptose-deficient mutants of *Escherichia coli*. J. Bacteriol. **117:**527-543.

25. **Korber, D. R., J. R. Lawrence, and D. E. Caldwell.** 1994. Effect of motility on surface colonization and reproductive success of *Pseudomonas fluorescens* in dual-dilution continuous culture and batch culture systems. Appl. Environ. Microbiol. **60:**1421-1429.

26. **Marshall, K. C.** (ed). 1984. Microbial adhesion and aggregation. Dahlem Konferenzen. Springer, Berlin.

27. **Miller, J.H. 1992.** A short course in bacterial genetics; A laboratory manual and handbook for *E. coli* and related bacteria. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

28. **Missiakas, D., J. M. Betton, and S. Raina.** 1996. New components of protein folding in extracytoplasmic compartments of *Escherichia coli*: SurA, FkpA and Skp/OmpH. Mol. Microbiol. **21:**871-884.

29. **Old, D. C., and J. P. Duguid.** 1970. Selective outgrowth of fimbriate bacteria in static liquid medium. J. Bacteriol. **103:**447-456.

30. **Parker, C. T., A. W. Kloser, C. A. Schnaitman, M. A. Stein, S. Gottesman, and B.W. Gibson.** 1992. Role of the *rfaG* and *rfaP* in determining the lipopolysaccharide core structure and cell surface properties of *Escherichia coli* K-12. J. Bacteriol. **174:**2525-38.

31. **Pilipcinec, E., T. T. Huisman, P. T. J. Willemsen, B. J. Appelmelk, F. K. de Graaf, and B. Oudega.** 1994. Identification by Tn*10* transposon mutagenesis of host factors involved in the biosynthesis of K99 fimbriae of *Escherichia coli*: Effect of LPS core mutations. FEMS Microbiol. Lett. **123:**201-206.

32. **Raetz, C. R.** 1996. Bacterial lipopolysaccharides: a remarkable family of bioactive macroamphiphiles, p. 1035-1063. In F. C. Neidhart. *Escherichia coli* and *Salmonella typhimurium*: Cellular and molecular biology. ASM Press, Washington, D. C.

33. **Rosenberg, M., E. A. Bayer, J. Delarea, and E. Rosenberg.** 1982. Role of thin fimbriae in adherence and growth of *Actinobacter caloaceticus* on hexadecane. Appl. Environ. Microbiol. **44:**929-937.

34. **Rosenberg, M., and S. Kjelleberg.** 1986. Hydrophobic interactions: Role in bacterial adhesion. In K. C. Marshall (ed). Advances in Microbial Ecology. **9:**353-393.

35. **Runnels, P. L., and H. W. Moon.** 1984. Capsule reduces adherence of enterotoxigenic *Escherichia coli* to isolated intestinal epithelial cells of pigs. Infect. Immun. **45:**737-740.

36. **Sambrook, J., E.F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

37. **Schagger, H., and G. von Jagow.** 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of the proteins in the range from 1 to 100 kDa. Anal. Biochem. **166:**368-379.

38. **Shapiro, J. A.** 1966. Chromosomal location of the gene determining uridine diphosphoglucose formation in *Escherichia coli* K-12. J. Bacteriol. **92:**518-520.

39. **Shea, C., L. J. Lovelace, and H. E. Smith-Somerville.** 1995. *Deleya marina* as model organism for studies of bacterial colonization and biofilm formation. J. Indus. Microbiol. **15:**290-296.

40. **Schnaitman, C. A., and J. D. Klena.** 1993. Genetics of lipopolysaccharide biosynthesis in enteric bacteria. Microbiol. Rev. **57:**665-682.

41. **Tsai, C. M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119:** 115-119.

42. **Wandersman, C., and S. Létoffé.** 1993. Involvement of Lipopolysaccharide in the secretion of *Escherichia coli* α–haemolysin in *Erwinia chrysanthemi* proteases. Mol. Microbiol. **7:**141-150.

43. **Welchenhan, D.** 1991. Fast recovery of DNA from agarose gels by centifugation through blotting paper TIG. **7:**109

44. **Weissborn, A. C., Q. Lui, M. K. Rumley, and E. P. Kennedy.** 1994. UTP:α-D-Glucose-1-Phosphate Uridylyltransferase of *Escherichia coli*: Isolation and DNA sequence of the *galU* gene and purification of the enzyme. J. Bacteriol. **176:**2611-2618.

45. **Williams, V., and M. Fletcher.** 1996. *Pseudomonas fluorescens* adhesion and transport through porous media are affected by lipopolysaccharide composition. Appl. Environ. Microbiol. **62:**100-104.

Chapter V

Identification of Tn*10* Insertions in the *dsbA* Gene Affecting *Escherichia coli* K-12 Adhesion to Polystyrene

ABSTRACT

Tn*10* insertion mutants of *E. coli* K-12 were selected for alterations in adhesion to a polystyrene surface (P. Genevaux, S. Muller, and P. Bauda, FEMS Microbiol. Lett. 142:27-30, 1996). Three adhesion mutants harbored the transposon in the *dsbA* gene, whose product, DsbA, catalyses folding of numerous extracytoplasmic disulfide bond-containing proteins. Adhesion, growth, type 1 fimbriae production, motility and type of lipopolysaccharide of the mutants were analyzed. All three mutants grew poorly, were non-motile, produced a very low amount of type 1 fimbriae, and exhibited changes in lipopolysaccharide structure. In spite of the pleiotropic character of the *dsbA* mutations, the loss of adhesion to polystyrene observed was mainly due to a reduced amount of type 1 fimbriae.

INTRODUCTION

Bacterial adhesion to non-biological surfaces in aqueous systems is the result of a complex interaction between the cell, the surface and the liquid phase. In this process, bacteria can employ several mechanisms to adhere to different surfaces under various environmental conditions. Proteins and polysaccharides were found to play a role in adhesion to inanimate surfaces. In *Staphylococcus epidermidis*, a proteinaceous surface antigen mediated attachment to polystyrene surface (32). Bacterial fimbriae, often associated with specific adhesion to biological surfaces, were found in some cases to play a considerable role in adhesion to inanimate surfaces (14, 15, 27). Adhesion of *Salmonella typhimurium* to various substrata was correlated with the presence of fimbriae (33). The presence of type 1 fimbriae conferred adhesion to *Escherichia coli* strains isolated from urinary track infection (14). An important correlation between the amount of type 1 fimbriae and adhesion was observed in various adhesion mutants of *E.coli* K-12 (10, 11). Flagella and motility were found to confer a selective advantage to *E. coli* K-12 and to *Pseudomonas fluorescens* during surface colonization (20, 10). Exopolysaccharides often appeared to be produced by attached bacteria (3, 30). Some types of lipopolysaccharide (LPS) were found to reduce adhesion to relatively hydrophobic surface (36). To better understand which bacterial structures are required for a specific surface and how different adhesive structures are interacting with each other, a genetic approach was chosen. The well-characterized *E. coli* K-12 was used as model for studying genes and membrane structures associated with adhesion to inanimate surfaces. Various Tn*10* insertion mutants deficient in adhesion to polystyrene were previously described (10, 11). This work presents Tn*10* insertions

localized in the *dsbA* gene, whose product is required for disulfide-bond formation in the periplasmic space (2). These insertions considerably affected adhesion to polystyrene (2). Cell membrane structure perturbations, such as dysfunctional motility, decrease in type 1 fimbriae biosynthesis or LPS alteration associated with these mutations were analyzed and their effects on adhesion are discussed.

MATERIALS AND METHODS

Bacterial strains, plasmid, and culture conditions

E. coli K-12 strains and their sources are listed in Table 1. Strains constructed for this work are mini-Tn*10* insertion derivatives of *E. coli* K-12 W3110 with altered adhesion properties (12). The ampicillin resistant plasmid pRI4 containing the *dsbA* gene under control of the arabinose promoter, was kindly provided by Arne Rietsch (Harvard Medical School, Boston, USA). For induction of the arabinose promoter, 0.1% arabinose was added to the culture medium. Cells were grown in static LB broth at 37°C or on LB agar plates (23). When necessary, media were supplemented with the following antibiotics: ampicillin, 100 µg/ml, and kanamycin, 30 µg/ml. The motility assay was carried out by applying 1 µl of a culture grown to a culture turbidity at 580nm of 0.6 (A_{580}=0.6) onto a soft agar plate (1% Tryptone, 0.5% Nacl, 0.35% agar). Diameters of growth were measured after 6 h incubation at 37°C.

Table 1: *E. coli* K-12 strains used in this study.

Strains	Revelant genotype[a]	Phenotype		Source or reference
		Motility[b]	Haemagglu-tination[c]	
HB101	F⁻ *lacY1 recA13* Δ*fim*	NT	-	(4)
W3110	F⁻ λ⁻ IN(*rrnD-rrnE*)1 *rph*-1	+	+	(1)
BGF4	*fimA*	+	-	(10)
BGA1	*dsbA* 518	-	-	this study
BGA4	*dsbA* 593	-	-	"
BGA5	*dsbA* 673	-	-	"

[a] For all the W3110::Tn*10* derivative strains, the name of the mutation referred to the nucleotide position of the mini-Tn*10* in the DNA nucleotide sequence corresponding to the Z50423 GenBank accession number.
[b] Determined by growth zones on soft agar plates and by observation under phase contrast microscopy.
[c] Ability to agglutinate rabbit erythrocytes after 5 min.

DNA manipulations

Isolation of plasmid DNA, transformation of $CaCl_2$-treated cells and all other basic DNA procedures were carried out essentially as described by Sambrook *et al.* (28). Restriction endonuleases were used according to the manufacturer's specifications (New England Biolabs).

Inverse PCR

For localization of the mini-Tn*10* insertion mutations in the *E. coli* chromosome, inverse PCR was carried out. Two oligonucleotides annealing closely together at the IS*903* of the mini-Tn*10* derivative 104 (Km^r) (17) were prepared (primer I: 5'-TTA CAC TGA TGA ATG TTC CG-3', primer II: 5'-GTC AGC CTG AAT ACG CGT-3'). Chromosomal DNA of the mutant strains was isolated and digested by either *Ava*II or *Hae*II. These restriction enzymes did not digest the region between the 3' end of primer II and the end of the IS*10*. DNA restriction fragments were then circularized. Subsequently, PCR using the circular fragments was carried out with 50 pmol of each primer. The reactions were run for 1 min at 96°C and for 30 cycles each consisting of 10 sec at 96°C, 30 sec at 55°C, and 2 min and 30 sec at 65°C. PCR products were extracted from agarose gels by centrifugation through blotting paper and purified by phenol/chloroform/isoamyl alcohol (50:48:2 by volume) extraction.

Nucleotide sequencing

Nucleotide sequencing was carried out using the Thermo Sequenase dye-terminator cycle sequencing kit from Amersham, with primer I as sequencing primer and double stranded PCR DNA fragments as template. Analyses were carried out using the 373A semi-automated DNA sequencer of Applied Biosystems/Perkin-Elmer. DNA sequences were checked for homology in the Genbank library.

Adhesion assay

Adhesion experiments were carried out using polystyrene microplates as previously described (12). Briefly, 20 µl of a cell culture in LB (A_{580}=0.70) were pipetted into the wells of a polystyrene microtiter plate containing 200 µl of fresh LB medium, and were allowed to grow and adhere for 2h, 5h, 8.5h, 13h, and 20 h respectively at 37°C. The optical density of the cultures in the wells was then measured (A_{580}). Unbound cells were removed and adhered cells were fixed for 30 min at 80°C. Subsequently, adhered cells were stained with crystal violet

(0.1%), and decolouring solution (ethanol/acetone, 80%: 20%) was added. The amount of eluted stain was estimated photometrically at 580 nm

Spot blot analysis

Bacterial cells were cultured at 37°C in static LB medium to A_{580}=0.70, collected by centrifugation (30 sec at 7 000 x *g*) and resuspended in phosphate-buffered saline (136.9 mM NaCl, 2.7 mM KCl, 10.0 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7.2). Serial dilutions of cell aliquots were spotted onto 0.2 μm nitrocellulose membrane paper, using the Bio-Dot apparatus from Bio-Rad. Next, the membranes were incubated with polyclonal rabbit antibodies directed against type 1 fimbriae, kindly provided by M. Dho-Moulin (INRA. Tours, France). The blots were then incubated with goat anti-rabbit conjugated to horse radish-peroxidase as secondary antibodies. Detection was carried out using the ECL kit from Amersham.

Haemagglutination

The capacity of bacterial cells to express a D-mannose-binding phenotype was assayed by their ability to agglutinate rabbit erythrocytes. Briefly, 50 μl of an aliquot of bacterial cells in phosphate-buffered saline (A_{580}=10.0) plus 50 μl of 5% erythrocytes in KRT solution (128.3 mM NaCl, 5.1 mM KCl, 1.3 mM $MgSO_4.7H_2O$, 2.7 mM $CaCl_2.2H_2O$, 10 mM Tris-HCl, pH 7.4) were mixed on glass slides at room temperature, and time until agglutination occurred was measured.

Isolation of periplasmic extract

Bacterial cells cultures (A_{580}=0.7) were washed twice in phosphate-buffered saline and concentrated to an A_{580}=40.0 in STE buffer (20% sucrose, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA). Next, EDTA (5 mM final concentration) and lysozyme (50 μg/ml final concentration) were added. Cells were incubated for 2 h at 4°C. The supernatant fractions, containing periplasmic extracts, were then collected after centrifugation for 5 min at 10000 *g*.

Protein techniques

SDS-PAGE was carried out essentially as described by Laemmli (22). Periplasmic extracts were separated by gel electrophoresis using 15% polyacrylamide gel. Following gel electrophoresis, proteins were transferred onto 0.45 μm nitrocellulose membrane, as described

by Krone *et al.* (21). Monoclonal rabbit antiserum raised against DsbA, kindly provided by A. Rietsch (Harvard medical school, Boston, Massachusetts, USA) was used to detect the DsbA protein.

Outer membrane preparation

Bacterial cells (A_{580}=0.7) were washed with phosphate-buffered saline, and concentrated to A_{580}=2.0 in a solution containing 50 mM Tris and 2mM EDTA (pH 8.5). Samples were frozen and thawed three times and sonicated three times for 10 sec. Cell debris were collected by centrifugation (30 min at 13 000 x *g*, 4°C) and the pellets containing outer membrane fractions were resuspended in 100 ml of phosphate-buffered saline. Proteinase K (1 mg/ml) was added and samples were incubated for 1 h at 60°C.

Gel electrophoretic analysis and silver staining of LPS

For analysis of LPS, outer membrane preparations were analyzed by gel electrophoresis using 18 % polyacrylamide gel and a (SDS)-tricine buffer system (29). Silver staining of the LPS was carried out as described by Tsai and Frasch (34).

RESULTS

Isolation of *dsbA* mutants

E. coli K-12 W3110::Tn*10* insertion mutants deficient in adhesion to polystyrene surface were reported before (12). The precise position of the mutation in the chromosome of the adhesion mutants was determined by inverse PCR and DNA sequence analysis. Three mutants had an insertion in the *dsbA* gene, whose product catalyses the formation of disulfide bonds in the periplasm (Table 1). To investigate the effect of the mutations on the *dsbA* gene product, the periplasmic fractions of the mutants were analyzed by SDS-PAGE. Following electrophoresis, proteins were transferred onto a nitrocellulose membrane and probed with an anti-DsbA antibody (Figure 1). DsbA was not detected in the mutants BGA1 and BGA4. For the mutant BGA5 a periplasmic product of an estimated molecular mass of about 20 kDa, which is about 2 kDa smaller than the wild type protein was observed. This band also had a lower intensity than the Dsba band of the parent strain. The position of the mutation in BGA5

(68 bp before the end of the *dsbA* structural gene), might result in a less stable C-terminally truncated product, lacking the 22 C-terminal amino acids produced in this mutant.

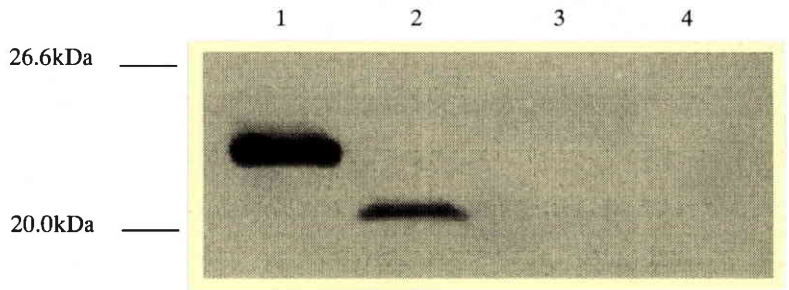

Fig. 1. DsbA protein in adhesion mutants. Periplasmic extracts were analyzed by 15% SDS-PAGE, transferred to nitrocellulose, and probed with anti-DsbA antibody: W3110 parent strain (lane 1), BGA5 *dsbA* (lane 2), BGA1 *dsbA* (lane 3), and BGA4 *dsbA* (lane 4).

The *dsbA* mutants have reduced adhesion and growth

The adhesion properties exhibited by the *dsbA* mutants were analyzed. Adhesion and growth were measured at various times after inoculation into polystyrene microplates. All the *dsbA* mutants were strongly affected in their ability to adhere to the polystyrene surface. Two groups of mutants could be distinguished on the basis of adhesion and growth characteristics. A first group, containing the mutants BGA1 and BGA4, and a second group containing the mutant BGA5. Figure 2 shows the adhesion abilities of the parent strain W3110 and representatives from the two groups of adherents observed. The parent strain adhesion versus cell density is maximal for up to 13 h. Maximal adhesion was reached after 13 h and a slight decrease in adhesion was found after 20 h. Insertion mutants BGA1 and BGA4 exhibited weak and identical adhesion properties after 20 h in the microplate. The adhesion of the wild type strain W3110 was reduced to the same level as the *dsbA* mutants BGA1 and BGA4, when D-mannose (2.5% w/v) was added to the incubation medium during the adhesion assay (see Discussion). Furthermore, the *dsbA* mutants showed the same adhesion as the non-fimbriated BGF4 *fimA* mutant described previously (10; data not shown). Besides adhesion, growth was considerably reduced in these two mutants. The mutant BGA5 displayed very weak adhesion and was dramatically affected for growth when compared to the null mutants BGA1 and BGA4. This might be caused by a toxic effect of the truncated DsbA produced in this mutant (Fig. 1). When plasmid pRI4 containing the cloned K-12 *dsbA* gene, was introduced into each of these mutants, the ability to adhere and grow was restored for the *dsbA* null mutants, but still

weak for the mutant BGA5. In addition, except for the mutant BGA5, the growth alterations of the *dsbA* null mutants were not observed under shaken culture conditions (data not shown).

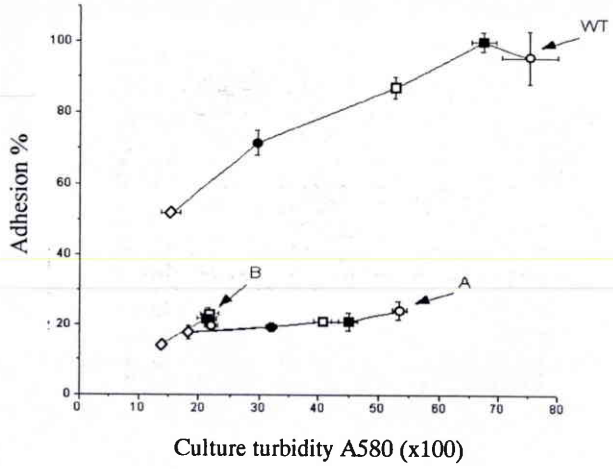

Fig. 2. Adhesion to polystyrene and growth properties of wild type and *dsbA* mutants: WT, W3110 parent strain; A, BGA4 *dsbA*; B, BGA5 *dsbA*. Adhesion and culture turbidity were measured 2 h (◇) 5 h (●), 8.5 h (□), 13 h (■) and 20 h (○) after inoculation. Error bars indicate the standard deviation based on triplicate measurements.

Mutations in *dsbA* affect motility, lipopolysaccharide structures and type 1 fimbriae biosynthesis

Extracytoplasmic proteins tend to contain disulfide bonds that contribute to their stability and in some cases, to their catalytic activity (24). The *dsbA* mutations are highly pleiotropic and have been found in many cases to block the folding of secreted proteins (24). To determine the effect of *dsbA* mutations on some outer membrane structures which might be important for adhesion, cell motility, LPS and fimbriation were analyzed.

To assay the motility capability, cells were applied onto soft agar plates and the ability to produce growth zones after 6 h of inoculation was determined. All the mutants were found to be non-motile.

To identify modifications in the LPS core in the *dsbA* mutants, outer membrane extracts were prepared, treated with proteinase K and then analyzed by tricine-(SDS) polyacrylamide gel electrophoresis. In Figure 3 a comparison of the silver stained LPS gel profile of the parent W3110 is made with those of the *dsbA* mutants. Most of the LPS of the parent W3110 strain migrated as a single broad band, whereas the three *dsbA* mutants produced

two bands, one migrating with the parent LPS (band 2) and one showing slower migration (band 1). The common band observed for the mutants and the parent strain corresponded to the rough (R) LPS exhibited by *E. coli* K-12 W3110 (19, 31).

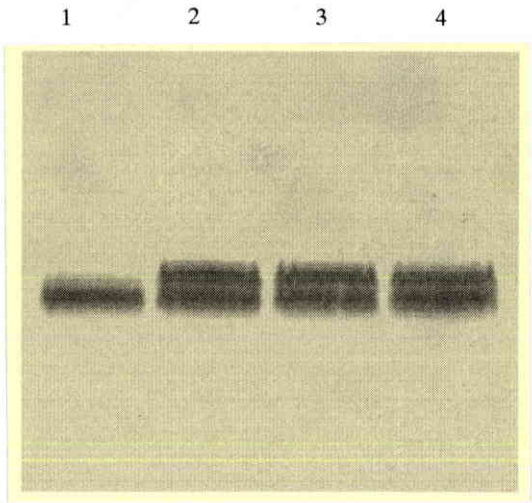

Fig. 3. Silver-stained gel of LPS. Proteinase K digestions of outer membrane extracts were separated on 18% polyacrylamide tricine gel, and silver stained. Lanes: 1, LPS from the parent W3110; 2, BGA1 *dsbA*; 3, BGA4 *dsbA* and 4, BGA5 *dsbA*.

To evaluate the amount of type 1 fimbriae produced by the *dsbA* mutants, spot blot analysis using anti-type 1 antibody and a haemagglutination assay were carried out. Cell suspensions were prepared and various dilutions were transferred to nitrocellulose and probed with an anti-type 1 antibody (Fig. 4).

Strain HB101 Δ*fim* was used as negative control. The two mutants BGA1 and BGA4 were found to produce less than 10% of the type 1 fimbriae than the parent strain. Strain BGA5, which produced a truncated DsbA protein and had a strong defect in growth, was found to produce even less fimbriae than the other mutants. As type 1 fimbriae contain a specific adhesin, FimH, that binds to mannose receptor at the surface of various red blood cells (see 18 for a review), the ability of the parent and the *dsbA* mutants to agglutinate rabbit erythrocytes was analyzed. No agglutination was observed for the mutants after 5 minutes, whereas the wild type strain showed a response in 30 seconds of contact with the red blood cells (Table 1).

The spot blot analysis and the haemagglutination test indicated that type 1 fimbriae biosynthesis was considerably affected by the *dsbA* mutations.

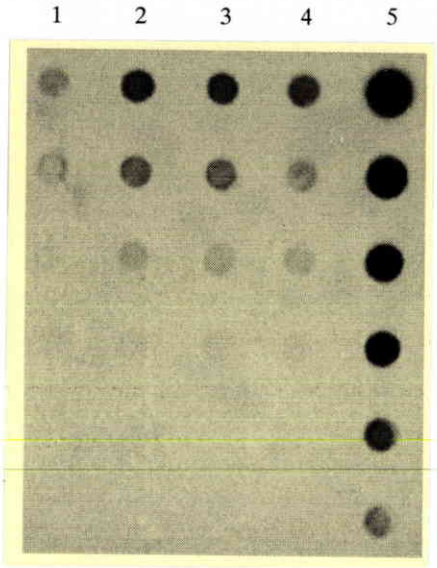

Fig. 4. Type 1 fimbriae production by spot blot analysis. Identical dilutions of whole cell suspensions were transferred to nitrocellulose and probed using anti-type 1 antibody. Lanes: 1, HB101 Fim$^-$; 2, BGA1 *dsbA*; 3, BGA4 *dsbA*; 4, BGA5 *dsbA*; 5, W3110 parent strain.

DISCUSSION

The well-characterized bacterium *E. coli* K-12 was used as model to identify genes and membrane structures associated with adhesion to inanimate surfaces. In this study, three Tn*10* insertion mutants which had inserts in the *dsbA* gene and which showed a reduced adhesion to polystyrene were studied. Considering the periplasmic localization of the DsbA protein, and its role in the folding of many secreted proteins, cell surface structures regarded as adhesives were analyzed. In these mutants, a reduced amount of type 1 fimbriae, a loss of motility, and a modified LPS accompanied the loss of adhesion observed.

The *dsbA* mutations have been found to affect fimbriae biosynthesis. Zhang *et al.* (37) showed that DsbA was critical for the biosynthesis of type IV fimbriae because of the essential role of disulfide bonds in the stability of the major structural protein. Adhesive P fimbriae of uropathogenic *E. coli* were not assembled by a strain that lacked the periplasmic disulfide isomerase DsbA (19). In that case, DsbA was required for disulfide bond formation in the Pap subunits and in the specific periplasmic chaperone PapD itself. The same study showed that type 1 fimbriae production was also affected by the absence of the DsbA protein. In contrast to the P fimbriae, the *dsbA* mutants were still able to assemble type 1 fimbriae, but in a lower

amount when compared with the parent strain. These differences might be explained by the absence of cysteines in the type 1 fimbriae periplasmic chaperone FimC (19).

Surface components such as fimbriae have been in some cases associated with adhesion to non-biological surfaces (10, 14, 33). In *S. typhimurium*, a significant correlation between the presence of fimbriae and the adhesion to various substrata was observed (33). Adhesion to polystyrene of *E. coli* strains isolated from urinary tract infections was correlated to the possession of mannose-sensitive fimbriae (14). Recently, Genevaux *et al.* (10) showed that type 1 fimbriae of *E. coli* K-12 strongly promoted adhesion to polystyrene, independently of the number of cells, of the age of the culture, and of the presence of functional flagella (10). In this study, the *dsbA* mutants were strongly affected in fimbriae production. These mutants showed the same adhesion as the non-fimbriated mutant BGF4 *fimA*, and the parent strain adhesion was reduced to the level of the *dsbA* mutants when D-mannose, the specific carbohydrate receptor of the type 1 fimbriae adhesin was added to the incubation medium. These data suggest that the reduced amount of type 1 fimbriae exhibited by the *dsbA* mutants was the main cause of the loss of adhesion properties observed. In addition, the decrease in attachment observed after 20 h for the parent strain might have been due in large part to a decrease in type 1 fimbriae expression by cells in the stationary phase of growth. Indeed, Dove *et al.* (9) showed that type 1 fimbriae were repressed, probably indirectly, by the RpoS stationary phase sigma factor of *E. coli*. Furthermore, the very low amount of type 1 fimbriae exhibited by the *dsbA* null mutants BGA1 and BGA4 may also explain the poor growth that was found for these mutants only in static culture. The presence of type 1 fimbriae was found to largely favor growth under this culture condition (5, 25). However, the cell factors regulating this response are not known as yet.

In addition to the loss of fimbriae, the *dsbA* mutants exhibited a non-motile phenotype. This is in accordance with Dailey *et al.* (6) who showed that *dsbA* mutants of *E. coli* failed to assemble the P ring of the flagella hook-basal-body because of the defective disulfide bond formation.

Motility is often cited as active participant in the adhesion process (7, 8, 13,14). In *P. fluorescens*, functional flagella permitted the rapid transport of the bacteria to the boundary layer. These cells achieved a higher final adhesion level (20). A recent adhesion study showed that, in *E. coli* K-12, motility conferred advantage during attachment to polystyrene substratum probably by increasing the chances of contact with the surface, whereas no evidences for an involvement of the flagellum itself in adhesion was observed (10). In a similar way, the loss of motility exhibited by the *dsbA* mutants could have influenced the adhesion patterns. However,

the strongly reduced amount of type 1 fimbriae produced by these mutants did not permit us to observe this effect.

Modifications in LPS structures were observed in the *dsbA* mutants. The absence of DsbA might affect activity of several modification enzymes in the periplasm, resulting in a partial modification of the LPS. The appearance of the so far unidentified upper band in the *dsbA* mutants suggested additional component(s) on the LPS core. Klena *et al.* (19) showed that a similar upper band could be observed in some *E. coli* K-12 strains, as a result of the transfer, in the periplasmic side of the inner membrane, of a single O antigen sugar (N-Acetyl-glucosamine) to the LPS core. Carriers that may be required to move the LPS through the periplasm have not yet been detected, and the manner that DsbA is acting in this process has yet to be described (26). Further investigations are necessary to determine the chemical structure of band 1 in the *dsbA* mutants.

In some cases, LPS has been associated with adhesion to inanimate surfaces, but its precise contribution in this process is not yet clear. In *S. typhimurium*, the presence of LPS was also found to reduce adhesion to air-water interface and to reduce cell surface hydrophobicity (15). In *P. Fluorescens*, an increased attachment to polystyrene associated with the attenuation or lack of the antigen O was observed (36). In *E. coli* K-12, mutations in genes affecting LPS core assembly induced a decrease in adhesion to polystyrene. However, the pleiotropic effect of the LPS mutations on type 1 fimbriae biosynthesis was found to be the major cause of the decrease in adhesion, whereas no evidence for a direct role of LPS in adhesion could be observed (11). In the case of the *dsbA* mutants, no evidence for an effect of the added band 1 on adhesion to polystyrene could be noticed.

This work further demonstrates the pleiotropic effect of the *dsbA* mutations in *E. coli*, and the major role of type 1 fimbriae in adhesion to polystyrene surface.

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REFERENCES

1. **Bachmann, B. J.** 1972. Pedigrees of some mutant strains of *Escherichia coli* K-12. Bacteriol. Rev. **36:** 525-557.

2. **Bardwell, J. C., K. McGovern, and J. Beckwith.** 1991. Identification of a protein required for disulfide bond formation *in vivo*. Cell **67:**581-589.

3. **Boyd, A., and A. M. Chakrabarty.** 1995. *Pseudomonas aeruginosa* biofilms: role of the alginate exopolysaccharide. J. Indus. Microbiol. **15:**162-168.

4. **Boyer, H.W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**:459-472.

5. **Brinton, C. C., jr.** 1965. The structure, function, synthesis and genetic control of bacterial pili and molecular model for DNA and RNA transport in gram negative bacteria. Transactions of the New York Academy of Sciences. **27:**1003-1054.

6. **Dailey, F. E., and H. C. Berg.** 1993. Mutants in disulfide bond formation that disrupt flagellar assembly in *Escherichia coli*. Proc. Natl. Acad. Sci. **90:**1043-1047.

7. **DeFlaun, M. F., B. M. Marshall, E. P. Kulle, and S. B. Levy.** 1994. Tn*5* insertion mutants of *Pseudomonas fluorescens* defective in adhesion to soil and seeds. Appl. Environ. Microbiol. **60:**2637-2642.

8. **DeFlaun, M. F., A. Tanzer, A. L. McAteer, B. Marshall, and S. B. Levy.** 1990. Development of an adhesion assay and characterization of an adhesion-deficient mutant of *Pseudomonas fluorescens*. Appl. Environ. Microbiol. **56:**112-119.

9. **Dove, S. L., S. G. J. Smith, and C. J. Dorman.** 1997. Control of *Escherichia coli* type 1 fimbrial gene expression in stationary phase: a negative role for RpoS. Mol. Gen. Genet. **254:**13-20.

10. **Genevaux, P., M. S. DuBow, B. Oudega, and P. Bauda.** The Role of Flagella and Type 1 fimbriae in *Escherichia coli* K-12 adhesion to inanimate surface. *Chapter III.*

11. **Genevaux, P., M. S. DuBow, B. Oudega, and P. Bauda.** Identification of Tn*10* insertions in the *rfaG*, *rfaP*, and *galU* genes involved in lipopolysaccharide core biosynthesis of *Escherichia coli* K-12, affecting adhesion to inanimate surface. *Chapter IV.*

12. **Genevaux, P., S. Muller, and P. Bauda.** 1996. A rapid screening procedure to identify mini-Tn*10* insertion mutants of *Escherichia coli* K-12 with altered adhesion properties. FEMS Microbiol. Lett. **142:**27-30.

13. **Graf, J., P. V. Dunlap, and E. G. Ruby.** 1994. Effect of transposon-induced motility mutations on colonization of the host light organ by *Vibrio fischeri*. J. Bacteriol. **176:**6986-6991.

14. **Harber, M. J., R. Mackenzie, and A. W. Asscher.** 1983. A rapid bioluminescence method for quantifying bacterial adhesion to polystyrene. J. Gen. Microbiol. **129:**621-632.

15. **Hermansson, M., S. Kjelleberg, T. K. Korhonen, and T. A. Stenström.** 1982. Hydrophobic and electrostatic characterization of surface structures of bacteria and its relationship to adhesion at an air-water surface. Arch. Microbiol. **131:**308-312.

16. **Jacob-Dubuisson, F., J. Pinkner, Z. Xu, R. Striker, A. Padmanhaban, and S. J. Hultgren.** 1994. PapD chaperone function in pilus biogenesis depends on oxidant and chaperone-like activities of DsbA. Proc. Natl. Acad. Sci. USA. **91:**11552-11556.

17. **Kleckner, N., J. Blender, and J. Gottesman.** 1991. Uses of transposons with emphasis on Tn*10*. Methods Enzym. **204:**139-180.

18. **Klemm, P.,** and **K. A. Krogfelt.** 1994. Type 1 fimbriae of *Escherichia coli*, p. 9-26. In P. Klemm (ed.), Fimbriae: Adhesion, genetics, biogenesis. and vaccines. CRC Press, Ann Arbor, Mich.

19. **Klena, J. D., and C. A. Schnaitman.** 1994. Genes for TDP-Rhamnose synthesis affect the pattern of lipopolysaccharide heterogeneity in *Escherichia coli* K-12. J. Bacteriol. **176:**4003-4010.

20. **Korber, D. R., J. R. Lawrence, and D. E. Caldwell.** 1994. Effect of motility on surface colonization and reproductive success of *Pseudomonas fluorescens* in dual-dilution continuous culture and batch culture systems. Appl. Environ. Microbiol. **60:**1421-1429.

21. **Krone, W.J.A., P. De Vries, G. Koningstein, A.J.R. De Jong, F.K. De Graaf, and B. Oudega.** 1986. Uptake of cloacin DF13 by susceptible cells: removal of immunity protein and fragmentation of cloacin molecules. J. Bacteriol. **166**:260-268.

22. **Laemmli, U.K.** 1970. Cleavage of structural proteins during the assembly of the haed of of bacteriophage T4. Nature. **227**:680-685.

23. **Miller, J.H. 1992.** A short course in bacterial genetics; A laboratory manual and handbook for *E. coli* and related bacteria. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

24. **Missiakas, D., and S. Raina.** 1997. Protein folding in the bacterial periplasm. J. Bacteriol. **179:**2465-2471.

25. **Old, D. C., and J. P. Duguid.** 1970. Selective outgrowth of fimbriate bacteria in static liquid medium. J. Bacteriol. **103:**447-456.

26. **Raetz, C. R.** 1996. Bacterial lipopolysaccharides: a remarkable family of bioactive macroamphiphiles, p. 1035-1063. In F. C. Neidhart. *Escherichia coli* and *Salmonella typhimurium*: Cellular and molecular biology. ASM Press, Washington, D. C.

27. **Rosenberg, M., E. A. Bayer, J. Delarea, and E. Rosenberg.** 1982. Role of thin fimbriae in adherence and growth of *Actinobacter caloaceticus* on hexadecane. Appl. Environ. Microbiol. **44:**929-937.

28. **Sambrook, J., E.F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

29. **Schagger, H., and G. von Jagow.** 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of the proteins in the range from 1 to 100 kDa. Anal. Biochem. **166:**368-379.

30. **Shea, C., L. J. Lovelace, and H. E. Smith-Somerville.** 1995. *Deleya marina* as model organism for studies of bacterial colonization and biofilm formation. J. Indus. Microbiol. **15:**290-296.

31. **Schnaitman, C. A., and J. D. Klena.** 1993. Genetics of lipopolysaccharide biosynthesis in enteric bacteria. Microbiol. Rev. **57:**665-682.

32. **Timmerman, C. P., A. Fleer, J. M. Besnier, L. DeGraaf, F. Cremers, and J. Verhoef.** 1991. Characterization of a proteinaceous adhesin of *Staphylococcus epidermidis* which mediates attachment to polystyrene. Infect. Immun. **59:**4187-4192.

33. **Stenström, T. A., and S. Kjelleberg.** 1985. Fimbriae mediated non-specific adhesion of *Salmonella typhimurium* to mineral particles. Arch. Microbiol. **143:**6-10.

34. **Tsai, C. M., and C. E. Frasch.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119:** 115-119.

35. **Weichenhan, D.** 1991. Fast recovery of DNA from agarose gels by centifugation through blotting paper TIG. **7:**109

36. **Williams, V., and M. Fletcher.** 1996. *Pseudomonas fluorescens* adhesion and transport through porous media are affected by lipopolysaccharide composition. Appl. Environ. Microbiol. **62:**100-104.

37. **Zhang, H. Z., and M. S. Donnenberg.** 1996. DsbA is required for stability of the type IV pilin of enteropathogenic *Escherichia coli*. Mol. Microbiol. **21:**787-797.

Chapter VI

Identification of Tn*10* Insertions in the *phnI*, *yhhT*, *hisT*, and *abc* Genes, and in the IS*186* Element of *Escherichia coli* K-12: Effect on Adhesion to a Polystyrene Surface

ABSTRACT

In the previous chapters, various Tn*10* insertion mutants with altered adhesion properties to a polystyrene surface are described. Herein, adhesion mutants harboring insertions in the *phnI*, *yhhT*, *hisT*, *abc* genes and in one of the three IS186 elements of the *E. coli* genome are presented. In addition to adhesion to polystyrene, type 1 fimbriae production, as well as the motility capability were analyzed. The results showed various degrees of alteration in these mutants.

INTRODUCTION

Studying *E. coli* K-12 adhesion to polystyrene surface, several Tn*10* chromosomal insertions associated with an adhesion-deficient phenotype were isolated (see *Chapters III, IV* and *V*). This chapter presents five adhesion mutants that harbored the mutation in the *phnI*, *yhhT*, *hisT*, and *abc* genes and in one of the three IS*186* of the *E. coli* genome (Table 1). The localization of the transposon in the *E. coli* chromosome was determined using inverse PCR and DNA sequence analysis (*Chapter III*). The growth conditions and the adhesion assay are detailed in *Chapter II*. The motility on soft agar plates and the ability to agglutinate rabbit erythrocytes were carried out as described in *Chapter III*. These mutants were affected in motility capability and/or in type 1 fimbriae biosynthesis. In contrast with the previous chapters, no evidence for an eventual role of these genes in fimbriae biosynthesis or motility were observed, and only one mutation was found in each of these genes. It is thus understandable that the mutations described here need to be more carefully studied before further conclusions can be drawn.

RESULTS AND DISCUSSION

Table 1: Bacterial strains used in this study.

Strains	Genotype[a]	Phenotype		GenBank accession number	Source or reference
		Haemagglu-tination[b]	Motility[c]		
W3110	F⁻ λ⁻ IN(*rrnD-rrnE*)1 *rph*-1	+	+		(2)
BGX1	*phnI* 5620	-	+	D90227	this study
BGX2	*yhhT* 1612	-	-	D90803	"
BGX3	*abc* 52884	-	+	U70214	"
BGX4	*hisT* 1411	+	+/-	X02743	"
BGX5	IS*186*	-	-		"

[a] For the W3110 derivative strains, the number of the mutation refers to the nucleotide position of the Tn*10* in the corresponding GenBank accession number.
[b] Agglutination after 5 min.
[c] Determined by the production of growth circles on soft agar medium.

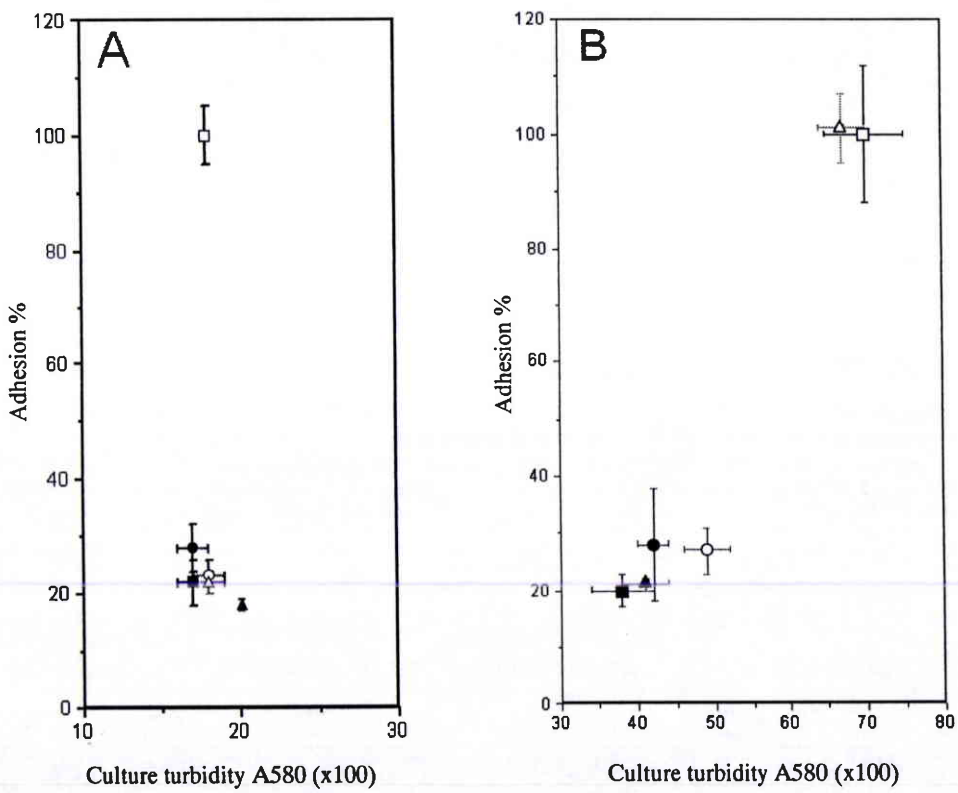

Fig.1. Adhesion properties to polystyrene microplates: Adhesion and culture turbidity were measured 2h (A) and 16 h (B) after inoculation. W3110 (□), BGX1 *phnI* (■), BGX2 *yhhT* (●), BGX3 *abc* (○), BGX4 *hisT* (△), BGX5 IS*186* (▲). Error bars indicated standard deviations based on triplicate measurements.

PhnI mutation

Mutant BGX1 harbors a Tn*10* insertion in the *phn* locus at 93.0 min in the *E. coli* map (Table 1). The *phn* (*psiD*) locus, encoding 14 Psi proteins (named PhnC to PhnP), is part of the Pho regulon. The members of the Pho regulon are phosphate-starvation-inducible genes mostly involved in the process of phosphorus assimilation. These genes are induced when inorganic phosphate, the preferred phosphorus source, is limiting for growth (9, 10). The *phn* (*psiD*) locus is involved in uptake and breakdown of phosphonate, an alternative phosphorus source in nature. The Tn*10* was localized in the *phnI* structural gene one of the *phn* genes required for the cleavage of the carbon-phosphorus bonds present in phosphonates. These genes are organized in an operon, and the mutational effect, as to be distinguished from effects due to polarity on downstream genes. In this case, the four genes downstream of *phnI* are also involved in catalysis (9). This mutant exhibited a very weak adhesion of about 80 % less than the parent strain after 2 h and 16 h of inoculation. Beside adhesion, growth was also affected after 16 h (Fig. 1). Motility was not affected, but the mutant was not able to agglutinate rabbit erythrocytes and therefore was affected in type 1 fimbriae production (Table 1) (6). This adhesion mutant exhibited the same characteristics as the *fim* mutants, and the defect adhesion and growth may be due to the loss of fimbriae (*Chapter III*). As described above, the *phn* genes are induced only when phosphate is limited (9). The growth conditions used in the adhesion assay are not phosphate-limited conditions (*Chapter II*). Furthermore, phosphonate utilization in *E. coli* K-12 is cryptic (10). Relations between the important defect in fimbriae production and the *phn* locus are thus difficult to establish, and further genetic characterizations are necessary.

YhhT mutation

In the mutant BGX2, the Tn*10* insertion was located at 36.0 min in the *E. coli* genome, 7 bp upstream of the *yhhT* structural gene encoding a hypothetical 38.5 kDa protein (Table 1). The *yhhT* gene is surrounded in the downstream region by the *pntAB* operon, encoding a pyridine nucleotide transhydrogenase that probably forms NADPH for anabolic purposes (4), and by a putative ethidium bromide resistance protein at about 400 bp in the upstream region. Except for the nucleotide composition, nothing is known about the localization or the function of this product. Homology with a hypothetical protein of *Haemophilius influenzae* (accession number: P44646) and with a putative membrane protein of *Streptococcus pneumoniae* (GenBank accession number: U80599) could be found. Structural analysis of this YhhT hypothetical protein showed 8 potential transmembrane regions, 5 intra-cytoplasmic regions

and 4 extra-cytoplasmic regions. The adhesion and growth properties of this mutant were considerably affected when compared to the wild type strain W3110 (Fig. 1). Motility assays and haemagglutination tests (Table 1) revealed that this mutant was non-motile and was considerably affected in the production of type 1 fimbriae, mostly responsible for the adhesion and growth deficiency in the culture conditions used (*Chapter III*). The relation between this mutation and the membrane alterations are not yet established.

abc mutation

Strain BGX3 harbored the insertion in an open reading frame named *abc* (*A*TP-*b*inding cassette), at 4.8 min in the *E. coli* chromosome (Table 1). The *abc* gene product, and its function, are as yet unidentified, but the deduced amino acid sequence reveled a single ATP-binding domain (1). The ABC transporters are divided into two groups; (i) the ABC importers, permeases involved in nutrient uptake; (ii) the ABC exporters, involved in the export of nonproteinaceous secreted products as well as extracellular proteins that cannot use the major secretion routes (5). The mutant BGX3 exhibited a very weak adhesion and was affected in growth (Figure 1) in the proportion of the *fim* mutants (*Chapter III*). Motility was not affected but type 1 fimbriae were considerably reduced (Table 1). Fimbriae alteration in this mutant is mostly responsible of the adhesion deficiency in this mutant. No involvement of ABC exporters or importers in type 1 fimbriae biosynthesis has yet to be described.

hisT mutation

In the mutant BGX4, the mini-Tn*10* insertion was found in the *hisT* structural gene at 50.2 min in the *E. coli* genome (Table 1). The *hisT* gene encodes a pseudouridine synthetase I, which catalyzes formation of pseudouridine residues at position 38, 39 and 40 in the anticodon stem and loop of at least 30 tRNA isoaccepting species, including $tRNA^{His}$ (11). This is assumed to be the cause of the reduction in the general rate of translation observed in *hisT* mutants (7). Mutant BGX4 adhesion was 80 % inferior to the parent adhesion after 2 h and identical by 16 h after inoculation (Fig. 1). Fimbriae production was normal, whereas the motility was affected but not completely interrupted (Table 1). This result is in accordance with the fact that motility confers advantage in the first period of the adhesion assay (*Chapter III*). The relationship between flagella biosynthesis and the *hisT* mutation are not, as yet, known. However, a FlhC-FlhD consensus-like regulatory sequence is present directly upstream from *hisT*. Tsui *et al.* (8), observed that *hisT* transcription was not *flhC* or *flhD* dependent, but was increased in *flhC* and *flhD* mutants. The alteration in motility could have also been affected

indirectly through one of the multiple effects of *hisT* mutations on cellular metabolism or gene regulation. Further investigations are required to understand this effect.

IS*186* mutation

The mutant BGX5 harbored the Tn*10* insertion in one of the transposable elements, IS*186*, in the *E. coli* genome. *E. coli* K-12 W3110 posseses three copies of IS*186* in its genome: IS*186*A at 0.3 min; IS*186*B at 13.1 min and IS*186*C at 54.1 min (3). The Tn*10* insertion was located at the Arginine 328 of the 370 amino acid hypothetical protein of the IS*186*. The IS*186* (A, B, or C) of this mutant, in which the transposon was inserted, has not been identified. This strain had "*fim* mutant-like" adhesion and growth profiles (Fig. 1), was not able to agglutinate rabbit erythrocytes and was non-motile (Table 1). No relation has been found yet between the IS*186* elements and fimbriae or flagella biosynthesis. The adhesion deficient phenotype can also be due to polar effect of the mutation on some adjacent genes. Further investigations are required to identify in which of the three IS*186* elements of *E. coli* the transposon is inserted.

REFERENCES

1. **Allikmets, R., B. Gerrard, D. court, and M. Dean.** 1993. Cloning and organization of the *abc* and *mdl* genes of *Escherichia coli*: relationship to eukaryotic multidrug resistance. Gene. **136:**231-236.

2. **Bachmann, B.J.** 1972. Pedigrees of some mutant strains of *Escherichia coli* K-12. Bacteriol. Rev. **36:** 525-557.

3. **Birkenbihl, R. P., and W. Vielmetter.** 1991. Completion of the IS map in *Escherichia coli*: IS*186* positions on the *Escherichia coli* K-12 chromosome. Mol. Gen. Genet. **226:**318-320.

4. **Clarke, D. M., T. W. Loo, S. Gillam, and P. D. Bragg.** 1986. Nucleotide sequence of the *pntA* and *pntB* genes encoding the pyridine nucleotide transhydrogenase of *Escherichia coli.* Eur. J. Biochem. **158:**647-653.

5. **Fath, M. J., and R. Kolter.** 1993. ABC transporters: bacterial exporters. Microbiol. Rev. **57:**995-1017.

6. **Klemm, P., and K. A. Krogfelt.** 1994. Type 1 fimbriae of *Escherichia coli*, p. 9-26. In P. Klemm (ed.), Fimbriae: Adhesion, genetics, biogenesis. and vaccines. CRC press, Ann Arbor, Mich.

7. **Palmer, D. T., P. H. Blum, and S. W. Artz.** 1983. Effects of the *hisT* mutation of *Salmonella typhimurium* on translation elongation rate. J. Bacteriol. **153:**357-363.

8. **Tsui, H-C. T., P. J. Arps, D. M. Connolly, and M. E. Winkler.** 1991. Absence of *hisT*-mediated tRNA pseudouridylation results in a uracil requirement that interferes with *Escherichia coli* K-12 cell division. J. Bacteriol.**173:**7395-7400.

9. **Wanner, B. L.** 1994. Phosphate-regulated genes for the utilization of phosphonates in members of the family Enterobacteriaceae, p. 215-221. In A. Torriani-Gorini, E. Yagil, and S. Silver. Phosphate in microorganisms: Cellular and molecular biology. ASM press, Washington, D.C.

10. **Wanner, B. L.** 1996. Phosphorus assimilation and control of the phosphate regulon, p. 1357-1381. In F. C. Neidhart. *Escherichia coli* and *Salmonella typhimurium*: Cellular and molecular biology. ASM press, Washington, D.C.

11. **Winkler, M. E.** 1996. Biosynthesis of histidine, p. 485-505. In F. C. Neidhart. *Escherichia coli* and *Salmonella typhimurium*: Cellular and molecular biology. ASM press, Washington, D.C.

Summary

SUMMARY

The inherent tendency of bacteria to adhere and colonize natural and artificial surfaces is regarded as a fundamental aspect of bacterial pathogenesis and ecology. Bacterial colonization of non-biological surfaces in aqueous systems is the result of complex interactions between the cell, the surface and the liquid phase. This process usually takes place in two principal steps: the initial attachment of the bacteria to the surface, and the subsequent formation of a biofilm matrix. Numerous bacterial cell surface structures, such as flagella,pili, non-pilus proteins, lipopolysaccharides, and exopolysaccharides, were found, in some cases, to play a role in this process (*Chapter I*).

The interest in understanding cell surface components that mediate attachment to inanimate surface led us to focus on the identification of genes associated with this phenomenon. To this purpose, the well-characterized bacterium *Escherichia coli* K-12 and a polystyrene surface were used as model.

An adhesion assay, using polystyrene 96 well microtiter plates, was first developed. During this procedure, bacterial strains were able to grow and adhere simultaneously, and attached cells were measured after crystal violet staining (*Chapter II*). A random insertion mutagenesis was performed with a mini-Tn*10* in *E. coli* K-12 W3110, and the adhesion assay was used to identify mini-Tn*10* insertion mutants with altered adhesion abilities. One percent of the 7 000 mini-Tn*10* insertion mutants screened was found to exhibit a reduced adhesion (*Chapter II*).

The location of the mini-Tn*10* insertion within the affected gene in the isolated *E. coli* W3110::mini-Tn*10* adhesion mutants was determined using inverse PCR and nucleotide sequence analysis. The genetic characterization was complemented by analysis of cell surface structures such as fimbriae, flagella or lipopolysaccharides (*Chapter III, IV, V, VI*).

Twenty-five adhesion mutants harbored the insertions in the *fim* operon, responsible for type 1 fimbriation (*Chapter III*). In all cases, the *fim* insertion mutants did not produce fimbriae and exhibited a weak attachment to polystyrene. Adhesion of the wild type strain was strongly inhibited when D-mannose, a specific receptor of the type 1 fimbriae adhesin, FimH, was added during the adhesion assay. The data obtained indicated a major role of type 1 fimbriae and a direct involvement of the type 1 fimbriae specific adhesin FimH in adhesion to polystyrene.

Twelve insertions were located in genes involved in flagella biosynthesis (*Chapter III*). Two groups of adherents could be distinguished among these mutants: The first group of

mutants, which harbored the mini-Tn*10* insertions in class 2 genes of flagella biosynthesis, exhibited poor adhesion, were non-motile, non-flagellated, and produced about 30 % of the amount of fimbriae when compared to the wild-type cells. The second group, which harbored the mutation in the promoter region of the class 3 *fliC* gene encoding for the flagellin subunits, adhered better than the first group, produced abnormal flagella, had dysfunctional motility and about 70 % of the amount of fimbriae as compared to the parent strain. The loss of adhesion observed appeared to be mainly due to the attenuated fimbriation caused by the flagella mutations and to the dysfunctional motility.

Seven mutants harbored insertions in genes involved in lipopolysaccharide core biosynthesis (*Chapter IV*). Two insertions were located in the *rfaG* gene, two in the *rfaP* gene and three in the *galU* gene. These adhesion mutants were found to exhibit a deep rough phenotype and were affected, at different levels, in type 1 fimbriation and motility. The loss of adhesion exhibited by these mutants was mainly associated with altered type 1 fimbriae production and, to a lesser extent, with defective motility. Apart from the pleiotropic effect of these mutations affecting LPS on type 1 fimbriae and flagella biosynthesis, no evidence for an involvement of the LPS core itself in adhesion to a polystyrene surface could be found.

Three adhesion mutants with very low adhesion abilities had insertions at different positions in the *dsbA* gene, whose product, DsbA, catalyses folding of numerous extracytoplasmic disulfide bond-containing proteins (*Chapter V*). Type 1 fimbriae production, motility and the type of lipopolysaccharide exhibited by these mutants were analyzed. All three mutants were non-motile, produced a very low amount of type 1 fimbriae, and exhibited changes in lipopolysaccharide structure. In spite of the pleiotropic character of the *dsbA* mutations, the observed loss of adhesion to polystyrene appeared to be most correlated with the considerably reduced amount of fimbriae.

Chapter VI describes a group of five adhesion mutants that harbored insertions the *phnI*, *yhhT*, *hisT* and *abc* genes and in one of the three IS*186* elements of the *E. coli* chromosome. In all cases, the loss of adhesion was correlated either with reduced fimbriation and/or motility. The strain BGX1 harbored the mini-Tn*10* in the *phnI* gene, involved in uptake and breakdown of phosphonate, an alternative phosphorus source in nature. Although the loss of adhesion exhibited by this mutant was correlated with the affected fimbriae production, the role of the *phn* genes in fimbriae biosynthesis is not yet elucidated. The adhesion mutant BGX2 contained the insertion in the *yhhT* gene, a gene of unknown function. In this mutant, the loss of adhesion was associated with both fimbriae and flagella alterations. Strain BGX3 harbored the insertion in the *abc* gene of unknown function. This mutant was weakly adherent,

had normal motility, but produced a reduced amount of fimbriae. The relationship between the *abc* mutation and fimbriae biosynthesis are not yet known. In the mutant BGX4, the mini-Tn*10* was located in the *hisT* gene that encodes a pseudouridine synthetase I. This adhesion mutant was not affected in fimbriae production but exhibited a reduced motility. Further investigations are required to understand the relation between the *hisT* mutation, dysfunctional motility, and therefore reduced adhesion. The last mutant presented here had an insertion in a hypothetical protein located in one of the three IS*186* elements of the *E. coli* chromosome. This mutant was weakly adherent and was affected in both motility and fimbriae production. The real effect of this mutation on attachment, and therefore on flagella and fimbriae biosynthesis, demands further investigations.

In conclusion, this work demonstrates the importance of type 1 fimbriae, and especially of the specific adhesin, FimH, in adhesion to relatively hydrophobic surface. Furthermore, in spite of its importance in the early moments of the attachment process, flagella did not appear to act as direct polystyrene adhesins. In addition, apart from the pleiotropic effect of LPS mutations on type 1 fimbriae and flagella biosynthesis, no evidence for a direct involvement of the rough LPS of *E. coli* K-12 in adhesion could be found.

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