Type 1 fimbriae and Antigen 43

The interaction of two phase variable surface components of Escherichia coli

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Overexpression of the type 1 fimbrial adhesin, FimH

and

Correlation between type 1 fimbriae and Antigen 43

Ph.D. thesis

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Preface

This thesis represents part of the requirements for the Ph.D. degree at DTU. The work was carried out at Institute of Microbiology at the Technical University of Denmark under the supervision of Dr. Per Klemm. It was financed in part by the Danish Medical Research Council (Grant no. 9503048) and in part by Professor Catharina Svanborg from the Institute of Medical Microbiology in Lund, Sweden.

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Henrik Hasman

Summary

Type 1 fimbriae are thread-like surface structures found on most *Escherichia coli* strains as well as many other bacteria within the *Enterobacteriaceae* family. These organelles mediate specific adhesion to D- mannosides through the FimH protein. This specific interaction enables bacteria to adhere to mammalian epithelial cells within the intestine, bladder and urinary tract as well as to a broad variety of cells related to the immune system. All of the genes required to promote expression of type 1 fimbriae are located within the *fim* gene cluster. The transcription of the genes encoding the fimbrial structural components is controlled by a promoter located within an invertible DNA fragment referred to as the phase switch. This unique regulatory mechanism enables the cell to vary between a fimbriate and an afimbriate state.

One of the aims of the work presented in this thesis was to increase the amount of FimH produced by *E. coli*. This may prove advantageous for the development of vaccines directed against type 1 fimbriae-mediated pathogenicity. Furthermore, this protein has been shown to accommodate insertions, such as antigenic epitopes and metal binding peptides. In both cases, an increased amount of chimeric FimH within the fimbriae is desired.

Initial work examined the level of transcription from different positions within the *fim* gene cluster. Transcriptional fusions were constructed between the reporter gene *lacZ* and the *fimA, fimI, fimD* and *fimH* genes, respectively. This revealed a 50-fold difference in the level of transcription between the *fimA* and *fimH* genes. Therefore, a modified gene cluster was designed, where the *fimH* gene was transferred to a position either immediately in front of or behind the *fimA* gene. Northern blot analysis revealed that only the latter was able to give a signal with the *fimH* probe, however, this did not lead to any increase in the FimH content of the bacteria. Therefore, the levels of the fimbrial periplasmic chaperone, FimC, and the outer membrane protein, FimD, were also increased. This led to a significant increase in the FimH content within the cells, but not to any significant increase in the amount of FimH detected within individual fimbriae on the cell surface. In an attempt to overcome this, the levels of the two other minor components, FimF and FimG, were increased along with FimC and FimD. This approach resulted in an increase in FimH protein both within the cells (a factor 25) and within the fimbriae (a factor 10), an observation illustrated by immunofluorescence microscopy, receptor-blotting and adhesion assays.

As these experiments were performed in an *E. coli* K-12 strain, they were subsequently repeated in a possible vaccine candidate strain, the *E. coli* Nissle 1917. This work has shown that the results obtained in the K-12 strain were reproducible in the Nissle 1917 strain.

The characteristic group of self-exporting proteins called the autotransporters contains several known virulence factors, such as the IgA1 protease of *Neisseria meningitidis* and the Pet protein from *E. coli*. These proteins are described in the second part of the introduction. The adhesin AIDA-I of enteropathogenic *E. coli* as well as its *E. coli* K-12 homologue, Antigen 43, also belong to this group. AIDA-I is involved in diffuse adherence of enteropathogenic *E. coli*, but the biological function of Antigen 43 is yet to be elucidated. Antigen 43 is composed of two subunits and is present on the surface of bacteria in relatively high numbers (about 50,000/cell). It is transcribed from the *flu* gene as a preprotein that is subsequently cleaved into two

components, denoted the α and β subunits. The β subunit is the transmembrane part, to which the α subunit is non-covalently attached. The α subunit contains a functional domain which enables it to adhere to an α subunit of another cell, thus conferring auto-aggregation of the bacteria. The *flu* gene is also subject to phase variation. The second chapter of this thesis describes the characteristics of auto-aggregation mediated by Antigen 43. By constructing a *flu*$^-$ variant of the constitutive Antigen 43 expressing strain (the BD1302 strain), this work shows that auto-aggregation is completely abolished when the *flu* gene is knocked out, and that this phenotype can be restored solely by reintroducing the *flu* gene encoded on a plasmid. Furthermore, mixing the Antigen 43$^+$ and the Antigen 43$^-$ strains in equal amounts shows that Antigen 43 is required on both cell types in order to obtain complete auto-aggregation.

The influence of type 1 fimbrial expression upon the auto-aggregation phenomenon was also investigated. It is shown that the auto-aggregation phenotype is prevented when the cells are forced to express either type 1 fimbriae or other types of fimbriae. This is achieved without repression of Antigen 43 expression, thus suggesting that the loss of auto-aggregation occurs as a result of the physical presence of fimbriae on the cell surface. Finally, immunofluorescence microscopy employing Antigen 43-specific antibodies was used to show that introduction of the *oxyR* gene into the BD1302 strain represses Antigen 43 expression directly.

Due to the phase variation of Antigen 43, colonies with cells expressing this protein have a different morphology than colonies containing cells, which are not expressing Antigen 43. These, on the other hand, have been reported to express type 1 fimbriae. The former colony morphology form, called *frizzy* or Form 1, is large, flat, has a jagged edge and a rough surface. The latter colony morphology form, called *glossy* or Form 2, is small, high, has a circular edge and a smooth surface. In the third chapter it is shown that a third colony morphology type also exists, called Form 3, which is large, flat, has a circular edge and a smooth surface. This type is shown, by immunofluorescence microscopy employing specific antibodies directed against either FimA or Antigen 43, to consist of cells which produce neither type 1 fimbriae or Antigen 43. It is therefore concluded that the rough surface appearance of Form 1 colonies, but not the size variation, can be accounted for by Antigen 43 production. The size variation is suggested to be caused by phase variation of type 1 fimbriae on the cells and to be connected to an insertion in the *fimE* gene.

Dansk resumé

Type 1 fimbrier er tynde tråd-lignende strukturer, som findes på overfladen af de fleste *E. coli* stammer, samt på mange andre enterobakterier. Disse organeller medierer specifik adhæsion (via proteinet FimH) til D-mannosider og dermed også evnen til at hæfte til tarm-, mundhule- og urinvejs-epitelceller, samt til en lang række immun-relaterede celler i pattedyr. *fim* generne, der er nødvendige for at udtrykke type 1 fimbrier, er placeret som en kompakt gruppe på kromosomet. En promotor, som styrer udtrykket af de strukturelle *fim* gener er placeret på et vendbart stykke DNA. Dette bevirker, at udtrykket af type 1 fimbrier er underlagt fase variation mellem en fimbrieret og en ikke-fimbrieret fænotype, alt efter om promotoren vender i retning af *fim* generne, eller den den modsatte vej.

Et af formålene med det arbejde, der bliver præsenteret i denne afhandling var at øge den mængde af FimH, som *E. coli* er i stand til producere. Dette kan være en fordel, hvis man ønsker at konstruere vacciner rettet imod patogene type 1 fimbrierede bakterier. Ydermere er det tidligere blevet vist, at dette protein kan acceptere insertioner, som f.eks. antigene epitoper og metalbindende peptider. I begge disse tilfælde er det ønskeligt at kunne øge mængden af kimært FimH.

I første omgang blev transskriptionsniveauerne fra forskellige positioner i *fim* genklyngen undersøgt ved at konstruere transskriptionelle fusioner af reportergenet *lacZ* med henholdsvis *fimA, fimI, fimD* og *fimH*. Dette viste, at der var en 50 faktors forskel mellem transskriptionen af henholdsvis *fimA* og *fimH*. Derfor blev en modificeret *fim* genklynge konstrueret, hvor *fimH* genet blev placeret enten lige foran eller lige bagved *fimA* genet. Northern blot analyse viste, at kun sidstnævnte var i stand til at give en signalstyrke med en *fimH* probe som svarede til signalstyrken fra en *fimA* probe. Den forøgede transskription førte dog ikke til en forøgelse i FimH mængden i bakterierne. For at omgå dette problem, blev mængden af (gen dosis) af det fimbrielle transport maskineri forøget. Dette medførte at FimH koncentrationen i cellerne steg signifikant, men uden at øge mængden af FimH i selve fimbrierne. For at opnå dette, var det nødvendigt samtidig at øge mængden af to andre fimbrieproteiner (FimF og FimG) som formodentligt er med til at integrere FimH i organel strukturen.. Dette førte til en 25-faktors stigning af FimH indholdet i cellerne og en 10-foldig stigning af FimH i selve fimbrierne. Ekspressionssystemet var ikke blot funktionelt i en *E. coli* K-12 stamme, men kunne også reproduceres i en potentiel vaccinestamme (Nissle 1917).

Den karakteristiske gruppe af selv-eksporterende proteiner kaldet auto-transportere inkluderer flere kendte virulensfaktorer, så som IgA1 proteasen fra *Neisseria meningitidis* of Pet proteinet fra *E. coli*. Til denne gruppe af proteiner hører også adhæsinet AIDA-I fra *Enteropatogene E. coli*, samt dets *E. coli* K-12 homolog (Antigen 43), hvis biologiske funktion dog ikke er endelig afklaret. Antigen 43 består af 2 dele, der er til stede på overfladen af bakterierne i relativt stort antal (omkring 50,000/celle). Det er et produkt af *flu* genet og bliver syntetiseret som et præprotein, der efterfølgende bliver processeret til to separate peptid kæder, kaldet α- og β-kæderne. β- kæden er lokaliseret i ydermembranen hvortil α-kæden er associeret via elektrostatiske kræfter. α-kæden indeholder det funktionelle domæne, der via dets evne til at binde til en tilsvarende α-kæde på en nærliggende celle, er i stand til at forårsage autoaggregation. I kapitel 2 i denne afhandling beskrives de forskellige karakteristika ved autoaggregation forårsaget af Antigen 43. Det blev vist (ved at

konstruere en *flu*$^-$ variant af den konstitutive Antigen 43-udtrykker stamme BD1302), at autoaggregationen fuldstændigt kunne forhindres, når *flu* genet blev inaktiveret og at fænotypen kunne genskabes, ved at komplementere med *flu* genet i *flu*$^-$ mutanten. Endvidere blev det vist at autoaggregation skyldes intercellulær Antigen 43-Antigen 43 genkendelse.

Herudover blev samspillet mellem autoaggregation og type 1 fimbriering belyst. Type 1 fimbriering blev vist at kunne forhindre autoaggregraion og samme effekt kunne opnås med andre fimbrietyper. Dette skete uden at Antigen 43 ekspressionen blev undertrykt, hvilket antydede, at det var den fysiske tilstedeværelse af fimbrierne på overfladen af cellerne, der forhindrede autoaggregationen. Endelig blev det vist ved brug af immunofluorescence mikroskopi med Antigen 43 specifikke antistoffer, at komplementering med *oxyR* genet i BD1302 represserede Antigen 43 ekspressionen direkte.

På grund af Antigen 43's fase variation har kolonier, der indeholder Antigen 43-producerende celler, en forskellig morfologi i forhold til kolonier, der indeholder celler, som ikke udtrykker Antigen 43. Sidstnævnte udtrykker derimod type 1 fimbrier. Førstnævnte kolonier, kaldet *frizzy* eller Form 1, er store, flade, med ujævn kant og en ru overflade. Sidstnævnte, kaldet *glossy* eller Form 2, er derimod små, høje, har en cirkulær kant og en glat overflade. I kapitel 3 beskrives, at der faktisk også eksisterer en tredje type, kaldet Form 3, der er store, flade, har en cirkulær kant og en glat overflade. Celler fra denne type kolonier udtrykker hverken type 1 fimbrier eller Antigen 43 i nævneværdig grad. Der blev derfor konkluderet, at den ru overflade, men ikke størrelsesforskellen på kolonierne, skyldes ekspression af Antigen 43. Omvendt blev størrelsesvariationen forårsaget af type 1 fimbriernes fasevariation, der igen skyldes en insertion i *fimE* genet.

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Introduction

Escherichia coli (*E. coli*) possess at least three different phase variable components on its surface: fimbriae, flagella and a protein complex called antigen 43 (Ag43). Each of these surface components is a polymeric structure and each has a distinct potentially antagonistic biological role. Flagella are involved in motility, while fimbriae are involved in immobilizing the bacteria on host surfaces. Additionally, some fimbriae are involved in pellicle formation, which enables the bacteria to form a dense layer on the surface of liquid suspensions. Although the precise role of Ag43 is yet to be elucidated, its homology to an adhesin of diffuse adhering enteropathogenic *E. coli* strains suggests a role in adhesion of the bacteria, either to eukaryotic cells or to other bacteria. The scope of this introduction is to review the knowledge of two of the three surface components: fimbriae (PART I) with emphasis on type 1 fimbriae and antigen 43 (PART II). Each part is succeed by a reference list.

PART I

Fimbriae

A broad variety of distinct types of fimbrial gene clusters have been identified in *E. coli*. Some strains carry only one (e.g. K-12 strains, which only carry the genes coding for type 1 fimbriae), while others possess the genetic machinery for expressing two or more different fimbriae. Apart from type 1 fimbriae, which are expressed by virtually all *E. coli* strains, most other fimbriae seem to be connected to adherence in specific animal hosts only (de Graff and Gaastra, 1997). As these organelles serve to immobilize the bacteria on mucosal surfaces of the host and as many *E. coli* strains are pathogenic, it is easy to imagine a role for these fimbriae in the virulence of the bacteria. Indeed, some fimbrial systems like the 987P and K88 fimbriae are often encoded on plasmids that also contain genes, which code for virulence factors such as enterotoxins (de Graff and Gaastra, 1994). Several reports exist which link fimbrial gene expression with bacterial pathogenicity. Of these, the correlation of P fimbriae with certain urinary tract infection (UTI) syndromes has been clearly established (Kuhen *et al*., 1992). More than 90% of the *E. coli* strains isolated from patients with pyelonephritis expressed P fimbriae compared to only 7% of *E coli* isolated from the feces of healthy persons (Källenius *et al*., 1981). The ubiquity and adhesive activity of type 1 fimbriae in the *Enterobacteriaceae* have led to the suggestion that these rod-like organelles could also serve a purpose in pathogenicity. However, their ability to promote binding of *E. coli* to a wide variety of eukaryotic cells has, until recently, made the precise determination of their role in bacterial virulence difficult. In the following section, a short introduction to type 1 fimbriae will be provided, including the current

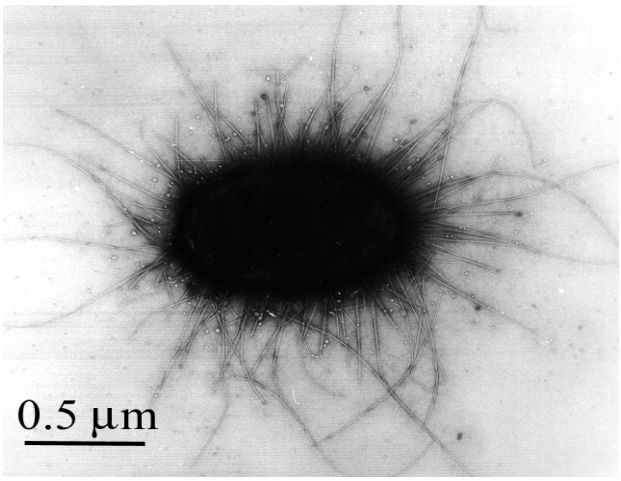

figure I.1. Electron microscopy image of a *E. coli* K-12 strain expressing type 1 fimbriae. Black bar indicates size scale.

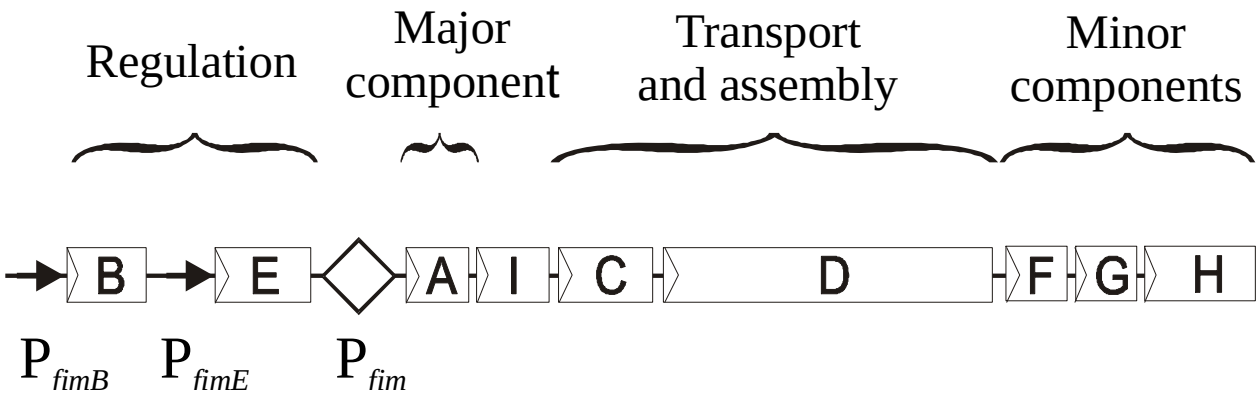

figure I.2. The *fim* gene cluster of *E. coli* K-12. Black arrows indicate promoters in front of fimB (P_{fimB}) and fimE (P_{fimE}). The invertible DNA fragment containing the *fim* promoter (P_{fim}) is represented by a white diamond.

understanding of their role in bacterial pathogenicity and their interaction with cells of the host's immune system. Finally, the prospects of employing type 1 fimbriae or fimbrial components as a vaccine delivery system will be discussed.

Type 1 fimbriae

Most enterobacteria, including *Escherichia coli*, have the ability to express different types of fimbriae on their surface (figure I.1). These filamentous organelles allow the bacteria to be immobilized in the appropriate environment and thus avoid being cleared from the host. Each type of fimbriae confers binding to specific receptors in various host compartments, as they each carry adhesive molecules with distinct specificity (for a review see Krogfelt and Klemm, 1994). In the case of type 1 fimbriae the adhesin subunit is called FimH and it is a lectin with specificity for D-mannose residues as part of host glycoproteins.

A type 1 fimbriated bacterium can carry up to 500 fimbriae on its surface. Each fimbria is a heteropolymer made out of at least 4 different proteins. The most abundant of these is FimA and it has been estimated that there are approximately 1000 FimA subunits per fimbria. The remaining1-2 % of the fimbrial structure consists of the three minor components: FimF, FimG and FimH. Two of these, FimF and FimG, are believed to form a complex with the FimH adhesin and thereby allow optimal presentation of FimH in the context of the fimbrial structure (Klemm and Christensen, 1987). At the base of each fimbria, one or more subunits of the outer membrane protein, FimD, are found. FimD serves as a polymerization platform and an anchor (also termed an "usher") for the fimbriae (Orndorff and Falkow, 1984; Klemm and Christensen, 1990).

The genes coding all these proteins are located in one locus at 97.6 min on the *E. coli* chromosome (Freitag and Eisenstein, 1983). These genes along with two other genes, *fimI* and *fimC*, are all transcribed from a single promoter ahead of the *fimA* gene (figure I.2). The *fimI* gene constitutes of an open reading frame that is expected to code for a protein similar to FimA, based on their high homology (30.4 % amino acid identity). An experiment employing the inducible T7 promoter system with this open reading frame suggests the presence of this protein on the fimbriae, but only in extremely small amounts (Klemm, personal communication). The *fimC* gene codes for the periplasmic chaperone FimC, which recognizes and binds to the structural components of the fimbriae, aiding in correct folding and assembly of the fimbrial subunits (Klemm, 1992; Jones *et al*., 1993).

Regulation

As already stated, a promoter located upstream of *fimA* drives the expression of the *fim* genes (Olsen and Klemm, 1994). This promoter is unique as it is located on a small, 314 base pair invertible DNA segment (Abraham *et al*., 1985). In this way, the cell is able to switch between a fimbriate and a non-fimbriate state (figure I.3), by inverting the DNA fragment, either pointing the promoter into the direction of the *fim* genes (denoted the ON orientation) or away from the *fim* genes (denoted the OFF orientation). The products of two other genes (called *fimB* and *fimE*), situated upstream of the switch, catalyze the physical inversion of the switch (Klemm, 1986; McClain *et al*., 1991, 1993; Gally *et al*., 1996). FimB and FimE are recombinases that specifically recognize a set of 9 bp sequences termed the left and right inverted repeats flanking the switch. The specificity of FimB and FimE for these repeats is different and gives rise to different effects on the switching rates (Gally *et al*., 1996). FimB turns the switch ON or OFF with approximately the same frequencies (10^{-3}-10^{-4} per cell per generation), regardless of growth conditions (Blomfield *et al*., 1991; McClain *et al*., 1991). FimE, on the other hand, turns the switch predominantly to OFF (frequencies up to 0.75 per cell per generation) when *E. coli* are grown in broth under shaking conditions or on solid media (Gally *et al*., 1993); it turns the switch ON when *E. coli* are grown statically (Pallesen *et al*., 1989; Stentebjerg-Olesen *et al*., 1999). This is most likely a result of the high selective pressure for the production of fimbriae in static broth due to the fact that fimbriation promotes pellicle formation. The formation of a surface layer (pellicle) may subsequently provide the cells with aerobic conditions and thus a strong growth advantage (Old and Duguid, 1970; Harris *et al*., 1990). Initiation of pellicle formation has been shown to rely on FimH and it is inhibited by α-methylmannoside (Harris *et al*., 1990). Type 1 fimbriae are the only fimbriae that have been shown to mediate formation of a pellicle.

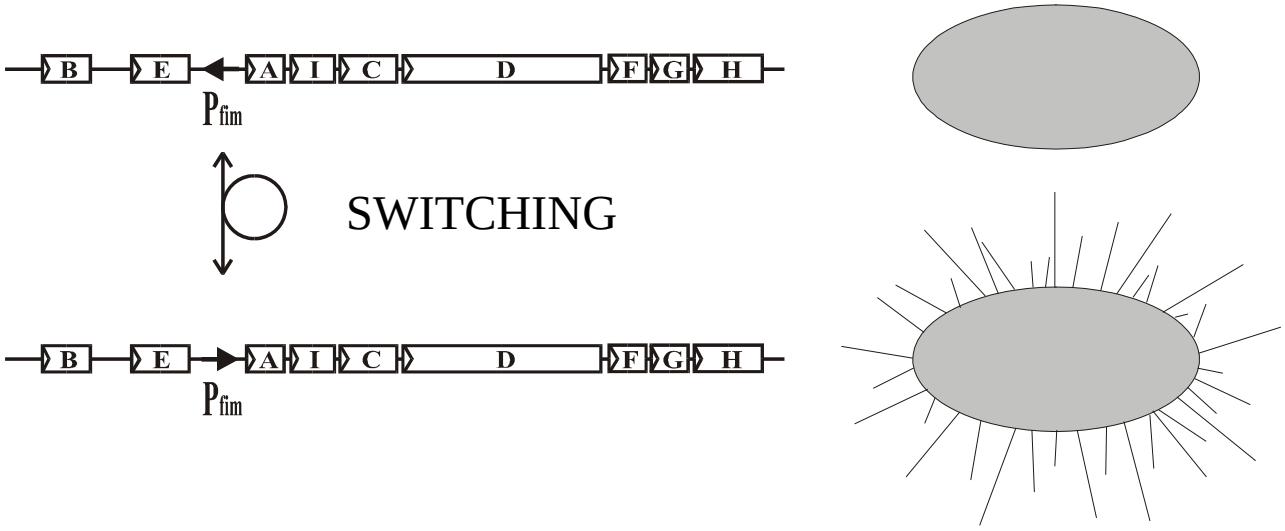

figure I.3. Simplified model of the phase switching event occurring in the *fim* gene cluster. The switching is controlled by the FimB and FimE recombinases as well as a broad variety of global regulators (see text). The direction of the *fim* promoter can either lead away from the *fim* genes (OFF) or through the *fim* genes (ON) indicated by black arrows.

The two recombinases and the phase switch are under the influence of a broad

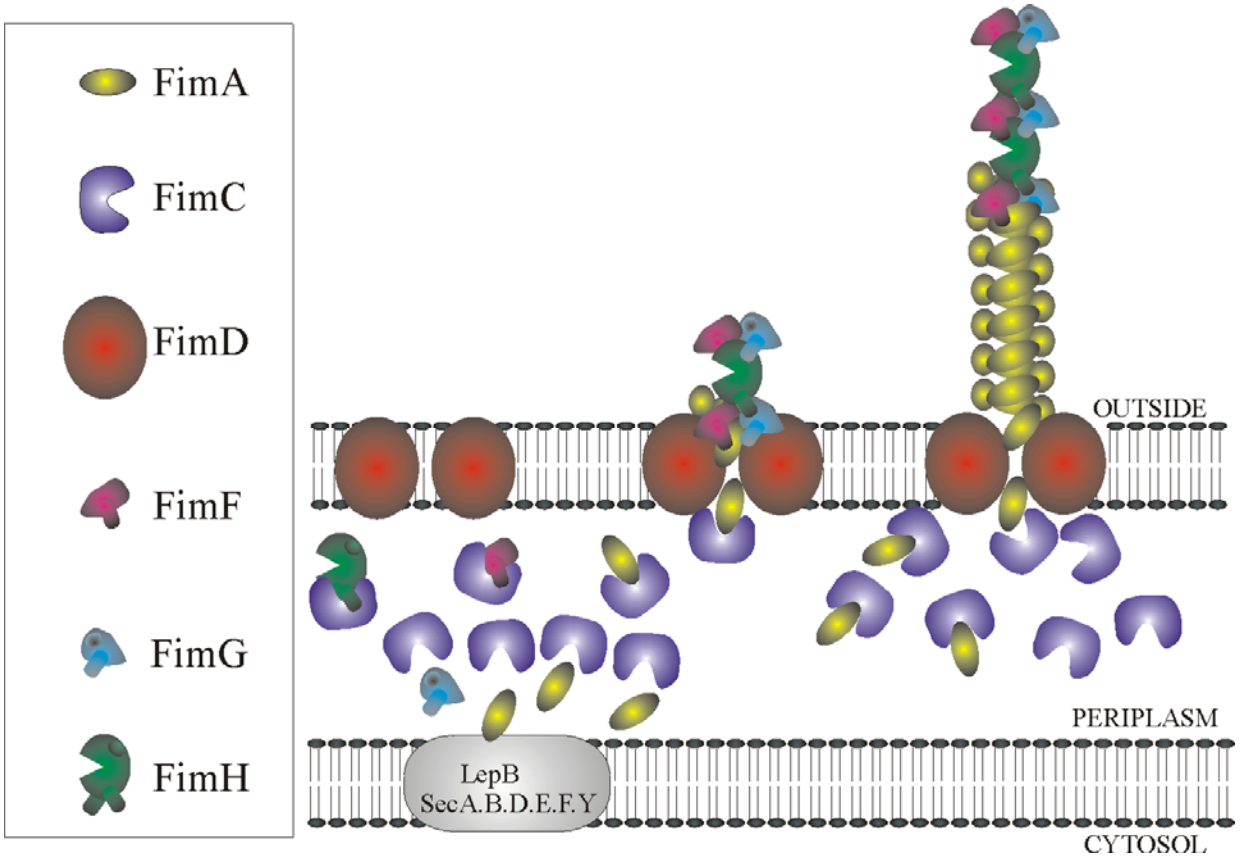

figure I.4. Model of the type 1 fimbrial biogenesis (note: The drawing is out of scale). See text for details.

variety of global regulators, which enable the cell to carefully control its degree of fimbriation according to environmental changes. Among the global regulators that affect fimbrial expression are Lrp (Blomfield *et al*., 1993; Gally *et al*., 1993 and 1994; Calvo and Matthews, 1994; Roesch and Blomfield, 1998), H-NS (Donato *et al*., 1997, Schembri *et al*., 1998), IHF (Eisenstein *et al*., 1987; Dorman and Higgins, 1987; Blomfield *et al*., 1997), RpoS (Dove *et al*., 1997) and LeuX (Newman *et al*., 1994; Ritter *et al*., 1997).

Biogenesis

Once the switch is turned in the ON orientation and the promoter initiates transcription of the *fim* genes, synthesis of the fimbrial proteins begins (figure I.4). Because all of the fimbrial proteins other than the recombinases possess classic signal peptides, they are transported across the inner membrane in a *sec* dependant manner (Dodd *et al*., 1984). As the nascent polypeptide enters the periplasm, the fimbrial structural proteins associate with the FimC chaperone due to the presence of a specific motif in the C terminal region that is recognized by FimC (Klemm and Christensen, 1987). FimC binding has dual purposes, protection of the fimbrial proteins from proteolytic degradation and the prevention of monomeric components aggregating within the periplasm, both of which would have a deleterious effect on the bacterium. Finally FimC is believed to be involved in transportation of the fimbrial structural components to the usher in the outer membrane and to deliver them in such a way that they can be correctly incorporated into the fimbrial organelle (Klemm *et al*., 1994 and 1995).

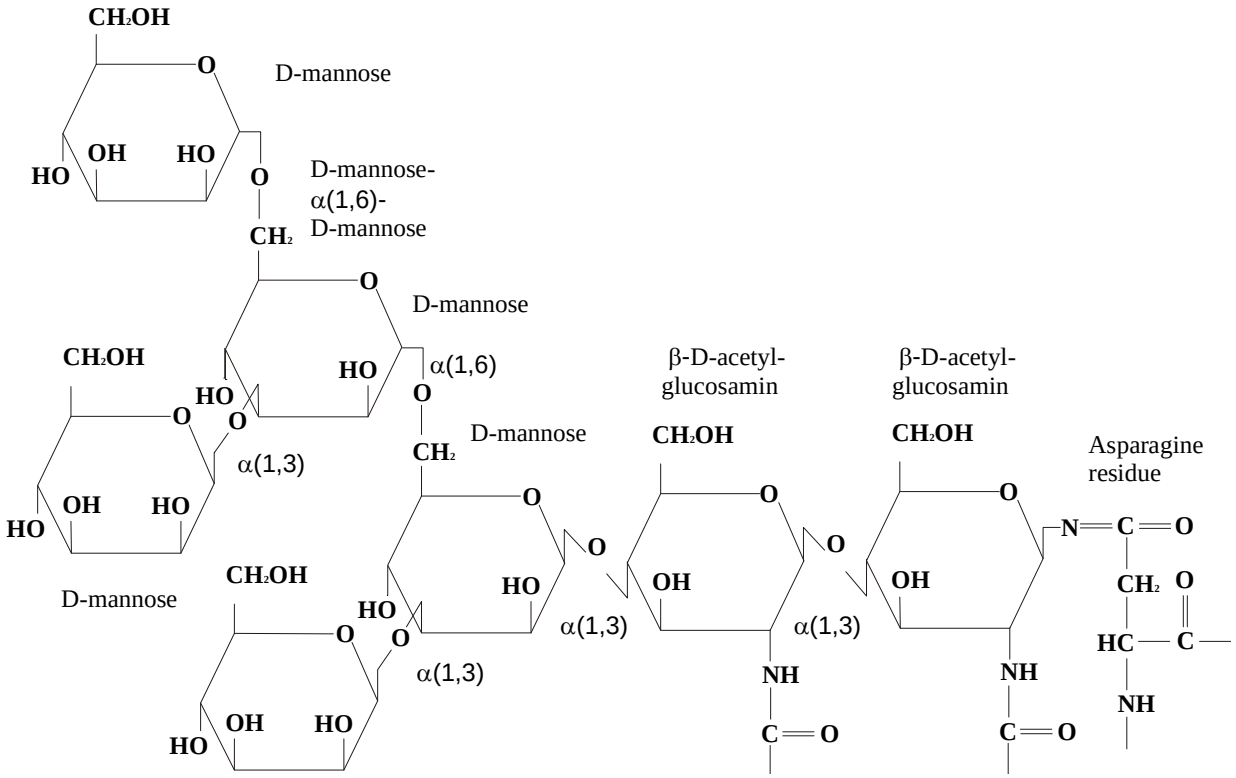

figure I.5. Schematic representation of a classical type 1 fimbrial receptor as described by Neeser *et al.* (1986). The solitary hydrogen atoms are left out for simplicity. The type of O-glycosidic bond between sugars is indicated next to the bonds.

In addition to the major component, the chaperone and the usher, initiation of fimbrial synthesis also requires the minor components: FimF, FimG and FimH (Klemm and Christensen, 1987; Russell and Orndorff, 1992). Deletions in one or more of the minor components results in changes in the length and number of fimbriae and specific deletion of the *fimH* gene results in loss of the adhesive phenotype. Divergent reports exist as to whether FimF and FimG are necessary in order to present FimH within the fimbriae: Klemm and Christensen (1987) report that cells are afimbriate and nonadhesive if neither FimF nor FimG are present whereas Russell and Orndorff (1992) report that such cells are both fimbriated and adhesive. In the wild type scenario, though, a more recent study (Jones *et al.*, 1995) reports that the three minor components are concentrated within a 16 nm long, thin fibrillar structure located at the tip of the fimbriae.

The adhesin

Initially, it was believed that a single fimbria consisted solely of the major structural component FimA (Brinton, 1965), but as better techniques emerged it became evident that at least three more proteins were to be found in this structure (Klemm and Christensen, 1987). The genes coding for these three proteins, *fimF*, *fimG* and *fimH*, are the most distal of the genes in the *fim* cluster (figure I.2). Soon after the discovery of these genes, others identified and characterized the adhesin as being the 28 kilo Dalton (kDa) protein FimH (Hanson and Brinton, 1988; Hanson *et al.*, 1988). Although these studies of Hanson and colleagues also indicated that the adhesin was present only at the tips of the fimbriae, it was subsequently shown that mannosylated BSA also bound to positions along the length of the fimbriae, thus indicating FimH was interspersed along the

figure I.6. Graphical illustration of the FimH protein with the three empiric domains indicated by different shades of gray. The N- and C-terminal ends are also indicated. Below are the relevant amino acid positions with the signal cleavage point arbitrary set as "0".

fimbrial shaft and not only at the tip (Krogfelt *et al*., 1990).

FimH from *E. coli* K-12 specifically recognizes mannosylated molecules (Figure I.5), but small changes in the amino acid composition can alter the receptor specificity of the adhesin. This enables the lectin to recognize protein structures like human plasma fibronectin and even small synthetic peptides lacking any sugar residues (Sokurenko *et al*., 1992). These minor changes are often seen in wild type variants of *E. coli* FimH, particularly among pathogenic strains isolated from urinary tract infections (Sokurenko *et al*., 1995 and 1997). Furthermore, evidence is now emerging that there actually could be more than one binding domain in each FimH molecule: one with high affinity for mono mannose residues and a second with high affinity for more complex (branched) mannose structures (Sokurenko, personal communication). It is assumed that both sites are only accessible at the tip (fibrillar), but not when FimH is residing in the fimbrial shaft.

This could account for the difference in binding affinity between naturally occurring fimbriae and fimbriae fragmentized by "freeze thawing" (Ponniah *et al*. 1991). These authors concluded that an additional, hidden, site in the fimbrial shaft could be exposed when the fimbriae are broken by ice-crystal formation, resulting in FimF, fimG and FimH complexes being exposed at the new fimbrial tips. This hypothesis is further supported by the fact that the binding characteristic of FimH from *Klebsiella pneumoniae* changes to the *E. coli* characteristic when it is placed on an *E. coli* type 1 fimbrial shaft and conversely, *E. coli* FimH behaves more like the FimH of *Klebsiella* when it is placed on a *Klebsiella* type 1 fimbrial shaft (Madison *et al*., 1994). Also, chimeric FimH molecules composed of the N-terminal half from *E. coli* (which contains the region conferring binding to D-mannose; see below) and the C-terminal half from *Salmonella typhimurium*, when inserted into a *S. typhimurium* fimbrial shaft, exhibit binding characteristics of the *S. typhimurium* adhesin (FimHS) and not the *E. coli* adhesin (FimHE) (Thankavel *et al*., 1999). This indicates that the fimbrial shaft participates in the receptor specificity, but not in the ability to recognize D-mannose per se.

The 3D crystal structure of FimH has not yet been published, so the chemical nature of D-mannose recognition is not known in detail. However, several attempts have been made to identify the amino acids directly involved in recognition. These include: (i) comparing FimH molecules from different clinical isolates and correlating their amino acid sequences with their distinct binding patterns (Sokurenko *et al*., 1994, 1995), (ii) analysis of the effect of linker insertions at several different positions in the *fimH* gene (Schembri *et al*., 1996) and (iii) construction of hybrid adhesins composed of FimH and FocH, the adhesin of F1C fimbriae (Knudsen and Klemm, 1998). Furthermore, amino acid residue ^{62}Ala in FimH from meningitis-associated *E. coli* is critical for the binding of

FimH to non-mannosylated collagens in a mannose-inhibitable manner (Pouttu *et al*., 1999). The binding to mannosylated targets, though, was not affected by this amino acid, suggesting a slightly different recognition domain of protein binding compared to sugar binding. Each of these studies suggested several amino acids or regions of FimH that could be involved in receptor recognition, either because there was a change in affinity to certain substrates or there was an abolishment of activity towards D-mannosylated compounds. In conclusion, the following picture emerges: The amino acids implicated in the binding were in the N-terminal 2/3 of the mature protein (figure I.6). The only amino acid position outside this N-terminal domain, which affected binding, was the terminal residue (position 279), where linker insertion resulted in loss of hemagglutination; a known characteristic conferred by type 1 fimbriae. This effect is more likely to be caused by interference with the recognition of the periplasmic chaperone or the other structural components of the fimbriae, rather than by alteration of the mannose-binding site.

Translational fusions (Thankavel *et al*., 1997) of parts of FimH to a carrier protein, MalE, revealed that there is sufficient structural information residing in the first 103 amino acids of FimH to confer binding to horseradish peroxidase (HRP), a mannosylated glycoprotein. These fusion proteins have not been thoroughly tested, however, so the binding to HRP may only reveal part of the binding motif, as amino acids following residue 103 have been shown to affect binding as well (see above).

Receptors for FimH

A large number of different glycoprotein receptors have been suggested to be a biological relevant target for type 1 fimbriated *E. coli*. These include the Tamm-Horsfall protein (Ørskov *et al*., 1980), secretory IgA (Wold *et al*., 1990), CD11/CD18 receptors of leukocytes (Gbarah *et al*., 1991), uroplakin Ia and Ib (Wu *et al*., 1996), CD48 on the surface of macrophages (Baorto *et al*., 1997) and laminin (Kukkonen *et al*., 1993). Along with these defined receptors, there also exists a tremendous number of reports that type 1 fimbriated bacteria adhere to a broad variety of eukaryotic cells, and for most of these the specific receptor remains to be discovered. Although the biological role of adhesion to most of these defined receptors is not yet known, a biochemical response in the target cells has been demonstrated in a few cases. In the next section, the most relevant of these will be presented.

Type 1 fimbriated bacteria and their interactions with the host

The function of type 1 fimbriae

Because many eukaryotic cells contain mannosylated surface molecules, it has been almost impossible to pinpoint unique compartments to which *E. coli* might exhibit tropism due to expression of type 1 fimbriae. This is in striking contrast to the strong tropism of for example P-fimbriated *E. coli* to the kidney due to the ability to recognize Gal-α(1-4)-Gal containing glycolipids present in high amounts in the kidneys. Due to the widespread occurrence of type 1 fimbriae among essentially all *E. coli*, even non-pathogenic fecal strains, it has often been speculated that the role of type 1 fimbriae is primarily within the normal life cycle of *E. coli* (Orndorff and Block, 1990). For example, it was shown (Block and Orndorff, 1990) that a type 1 fimbriated *E. coli* K-1 strain adhered far better to the surface of the oral cavity (the oropharynx) than their *fim*$^-$ counterparts. This suggested a role for type 1 fimbriae in the fecal-oral cycle, as the dose needed to pass the acidic barrier downstream of the oropharynx (i.e. the stomach) was far lower for the type 1 fimbriae bearing bacteria than the *fim*$^-$ strain. This hypothesis was further developed by an experiment

inoculating the same *fim*$^+$ and *fim*$^-$ strains into individual newborn mice, which were then placed into cages with non-infected mice. Seventy-two hours after inoculation, 90% of the littermates of the *fim*$^+$ inoculated mice, but only 10% of the *fim*$^-$ inoculated, mice contained the test bacteria in the oropharynx, suggesting the involvement of type 1 fimbriae in spreading from host to host (Block *et al*., 1992).

As receptors for type 1 fimbriae also exists on intestinal (Wold *et al*., 1988), vaginal (Falkowski *et al*., 1986; Venegas *et al*., 1995) and urothelial (Salit & Gotschlich, 1977; Wu *et al*., 1996) epithelial cells as well as in association with urothelial mucus (Ørskov *et al*., 1980; Chick *et al*., 1981), it is obvious that these organelles are involved in other events than host-to-host transmission. Due to the ability of type 1 fimbriated *E. coli* to binding mucus in the intestine and to the short generation time of *E. coli* it has been proposed that type 1 fimbriae enables the bacteria to maintain a high population size inside the host despite the constant loss of bacteria through feces (Abraham and Jaiswal, 1997).

The occurrence of type 1 fimbriated bacteria in infections has until recently been suggested to be secondary and as a consequence of certain fecal bacteria entering sterile compartments (e.g. the urinary tract) as a result of changed conditions caused by compromised hosts. However, due to a number of recent findings this picture has changed dramatically. It has now been clearly demonstrated that the FimH adhesin is critical for the ability of *E. coli* to colonize the urinary tract. *fimH*-null derivatives of highly urovirulent *E. coli* strains were shown to have greatly reduced capacity to initiate infection and to persist in the urinary tract when compared to the parental strains (Schaeffer *et al*., 1987). It has also been shown, that expression of type 1 fimbriae seriously enhanced *E. coli* virulence in urinary tract infections, at least in concert with expression of P-fimbriae (Connell *et al*., 1996). Recently, an explanation for this has been suggested (Mulvey *et al*., 1998) as only type 1 fimbriated uropathogenic *E. coli* were able bind to bladder epithelium through uroplakin Ia and Ib and to initiate exfoliation of the bladder cells resulting in destruction of the epithelial layer. Furthermore, only the type 1 fimbriated bacteria invaded the underlying cell layers, thus escaping the clearance along with the exfoliated epithelial cells.

Additionally, it has been found that even though FimH is structurally very highly conserved, only minor variations in the *fimH* gene can lead to important phenotypic differences. Indeed, it has been shown that there are naturally occurring allelic variants of FimH and those common to uropathogenic isolates appear to be especially effective for the pathogenic colonization of the urinary tract, as compared to those common to fecal isolates (Sokurenko *et al*., 1998).

The immune cells encountered by pathogenic E. coli and their connection to type 1 fimbriae

Apart from the broad variety of epithelial cell, which type 1 fimbriated bacteria has been shown to bind to, there also exist numerous examples of immune cells, to which these bacteria adhere to in a mannose sensitive manner. These cells include neutrophils, B-lymphocytes, mast cells and macrophages. In order to understand the implications involved in binding to these cells, a short introduction to the relevant immune cells will be given, based on Klein (1991), and their connection to type 1 fimbriae will be demonstrated. Furthermore, an inconclusive, simplified, model of the interaction of the immune system cells, centered on a macrophage, upon a bacterial invasion is shown below (figure I.7).

Neutrophils.

One of the first types of immune cells encountered by invading bacteria is the

neutrophil. These cells originate, like all the immune cells, from the bone marrow, wherefrom they enter the bloodstream and get transported to the capillaries. Here they first adhere to the vessel walls, force the vessel cells apart and enter the surrounding tissue (figure I.7). They respond upon chemical attractants, interleukins, produced by other cells of the immune system upon bacterial invasion and infiltrate in particular tissues that are in contact with the outside world: mucous membranes, the intestinal tract and the skin. The purpose of these cells is to scavenge the tissue for foreign objects and if these are encountered, they get phagocytized and degraded by lysosomes.

Neutrophils have been shown to possess at least nine different receptors for type 1 fimbriaed *E. coli* (Karlsson *et al*., 1996). Among these are: the complement receptor 3 (CD11b/CD18) (Gbarah *et al*., 1991), the nonspecific cross-reacting antigen (Sauter *et al*., 1993) and Lysosomal membrane glycoprotein (Lamb-1) (Karlsson *et al*., 1996). Several uropathogenic *E. coli* has been shown to adhere to neutrophils in a mannose-inhibitable manner, thus suggesting a role for type 1 fimbriae in this process (Lock *et al*., 1990). One of the two strains tested by Lock *et al.* was ingested by the neutrophils and killed by the combined action of lysosome-mediated release of enzymes into the phagocytic vacuole and an intracellular oxidative burst. This type of engulfment is different from the normal type seen during phagcytosis, which requires antibody-mediated opsonization and recognition of the bound antibody through F_c-receptors on the surface of the neutrophils. The other strain was not engulfed, but was killed by an extracellular oxidative burst on the surface of the cells. (Lock *et al*., 1990). However, the results of these experiments may be open to question, since the experiment did not utilize *fim*$^-$, isogenic, control strains. Furthermore, one of the strains (the engulfed) also exhibited mannose-insensitive adhesion indicating presence of other types of fimbriae. A more convincing experiment was later carried out (Tewari *et al*., 1993) employing purified nascent FimH to activate the neutrophils. This purified protein displayed the same mannose-inhibitable binding to the neutrophils as type 1 fimbriated bacteria, and it was adequate to trigger the oxidative response. FimH-coated fluorescent microspheres were even phagocytosed by the neutrophils, thereby mimicking the type 1 fimbriated bacteria. Recent investigations (Godaly *et al*., 1998) have shown, that P and type 1 fimbriated *E. coli* stimulate neutrophil migration across uroepithelial cell layers in vitro and recruitment of neutrophils in the urinary tract in vivo. This effect was related to the increased production of Interleukin-8 (IL-8) from the uroepithelial cells (figure I.7). IL-8 is a secreted agent involved in chemotaxic movement of several immune cells. This was not seen with their isogenic non-fimbriated counterparts, underlining the relationship between adhesion of *E. coli* to epithelia mediated by these fimbriae and the recruitment of neutrophils.

B-lymphocytes.

In blood, lymphocytes are, after neutrophils, the second-most numerous white blood cells. They fall into two main groups called T cells and B cells. These cells are mutually dependent on each other in order to elicit a strong specific immune response, as stimulation of the one requires lymphokines secreted by the other. The lymphokines stimulate proliferation of the correct clones of B-cells, subsequently leading to production of antibodies specific for the invading organism and thereby focusing the attention of the complete immune system upon the invader (figure I7). Lymphocytes can also be stimulated non-specifically without direct contact with cells. This mitogenic stimulation is often caused by polymers, which are able to bind to certain receptors on the lymphocyte surface. In contrast to the normal B- and T-

cell activation by antigens, where only a small subset of the total cells participate, mitogenic activation involves a large subset of the cells and thereby forces them to produce non-specific antibodies, many of which will not specifically aid in clearing the invader. Substances that are able to elicit a mitogenic response are bacterial LPS, flagella and a broad variety of lectins. B- and T-cells are part the systemic immune response and only bacteria that are able to enter the bloodstream or the lymphatic system will be encountered by these cells. However, their products (e.g. secretory IgA and interleukins) can influence immune cells in other compartments (e.g. within connective tissue and on mucosal surfaces).

B-lymphocytes also interact with type 1 fimbriated bacteria. Experiments involving type 1 fimbriae with and without the adhesin, FimH, show a clear difference in their ability to activate B-cells (Ponniah *et al*., 1989) Both types of fimbriae elicited a T-cell dependent response after 8 days, but only the FimH-containing fimbriae were able to produce a T-cell independent response by up to 75% of the B-cells after 3 days. This indicates that there was a mitogenic activation caused by the wild type fimbriae. Later experiments (Ponniah *et al*., 1992) provided evidence for immunoglobulin secretion from these FimH-activated B-cells, and this effect of type 1 fimbriae was inhibited by addition of α-methyl mannoside. The effect of this mitogenic activation of B-cells and the subsequent secretion of immunoglobulins is that a large portion of the immune cells meant to be ready for clearance of invading

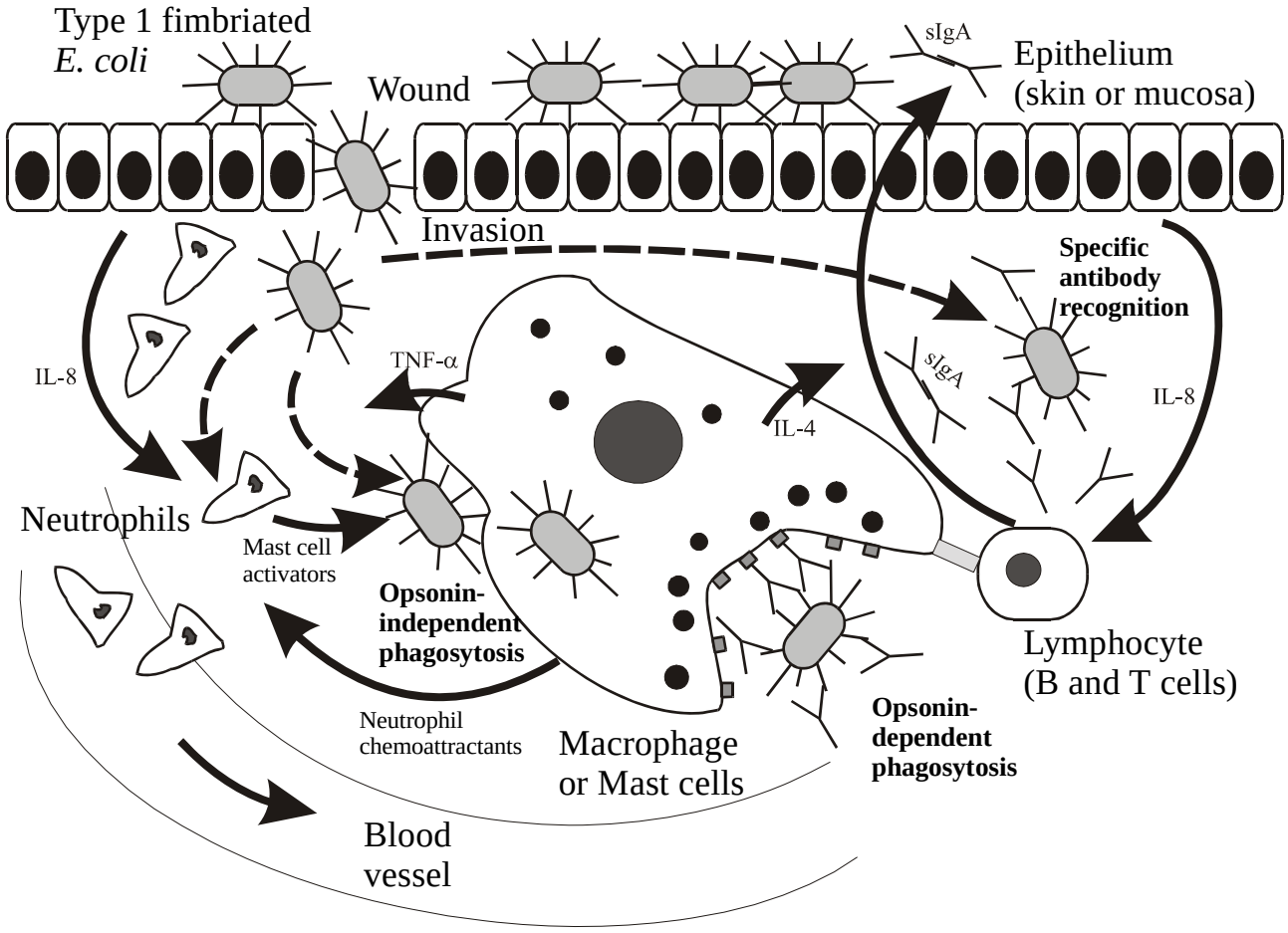

figure I.7. Simplified model (out of scale), based on the information given in text, in part describing the fate (broken lines) of a type 1 fimbriated bacteria upon invasion though the epithelium. Solid lines are indicating the flow of chemoattractants (interleukins, TNF-α etc.) between the different immune system cells.

microorganisms are activated non-specifically and are therefore not able to cope with the invader. This could be a way for invading type 1 fimbriated bacteria to cheat the immune system. In this way, the immune system might be deceived into a premature response without the required specificity. Interestingly, type 1 fimbriae were not able to activate T-cells, a feature associated with another mannose-specific lectin, concanavalin A, suggesting that a different type of receptor is recognized by the plant protein.

Mast cells.

Mast cells (reviewed by Abraham and Malaviya, 1997) are divided into two groups depending on the site of their appearance: mucosal-type and connective tissue-type mast cells. The function of these cells in infection and immunity are still unclear, but their presence in compartments commonly invaded by pathogens suggests a role in protection against infections (figure I.7). Mast cells have the capacity to release a wide range of proinflammatory substances, some of which are able to summon other types of immune cells and therefore their function could be to alert the immune system upon invasion of pathogens.

As mentioned above, the precise role for mast cells has not been elucidated, but their presence in the compartments prone to being invaded by pathogens implies a protective function. When type 1 fimbriated *E. coli* are incubated with mast cells, they are bound and phagocytized. This lead subsequently to killing of the bacteria by acidified vacuoles involving lysosomes and through the release of superoxide anions (oxidative burst), much like what is observed with neutrophils (Malaviya *et al*., 1994a and 1994b). As the bacteria are destroyed, they are degraded to small pieces (epitopes), which can be presented by the major histocompatibility complex (MHC) class I molecules on the surface of the mast cells. The MHC molecules function is to present the epitopes to the lymphocytes and thereby to transmit knowledge of the foreign material to the other immune cells, implying a role in the defense against invaders for these cells as well (Malaviya *et al*., 1996). Type 1 fimbriated bacteria have also been reported to stimulate the production of tumor necrosis factor (TNF-α) in mast cells (Dreskin and Abraham, 1999). Among other things, TNF-α also stimulates interleukin-I production, which acts as a chemotactic attractant of neutrophils (figure I.7).

Macrophages.

Macrophages are large phagocytic cells situated in the tissue surrounding capillaries. They are also abundant in areas of tissue injury or infection. Their function in infections is to ingest foreign particles and digest them by the use of lysosomes (figure I.7). But unlike neutrophils, which die when they are filled up with ingested material, macrophages continue the process of ingesting and digesting foreign material.

Again, type 1 fimbriated *E. coli* have also been reported to adhere to and get internalized by macrophages (Gbarah *et al*., 1993, Baorto *et al*., 1997). In contrast to the interactions of type 1 fimbriated bacteria with mast cells and neutrophils, the internalization of the bacteria by macrophages does not activate the host cells defense mechanisms, as the bacteria are not associated with the lysosomes and do not elicit an oxidative burst. The reason for this is supposed to be due to the internalization process, as the bacteria gain access to the macrophage through the receptor CD48 on the surface and not through antibody-mediated opsonization. In this way, the invading bacteria avoid being killed by gaining access to host compartments in which it can evade the normal host defense system.

Type 1 fimbriae as vaccine candidates

As described above, type 1 fimbriae play a central role in the contact between bacteria and the host. A considerable fraction of type

1 fimbriated *E. coli* have the capacity to cause infections and disease. Therefore, it seems reasonable to raise a protective immune response towards type 1 fimbriae in order to prevent these pathogens to establish themselves in the body. Several different attempts have been made over the last two decades to raise protective immune response against type 1 fimbriae, with mixed results. In the following section, these trials will be described and discussed. Furthermore, fimbriae as carriers of foreign epitopes will be presented.

Purified type 1 fimbriae as vaccines

The most logical and cost-effective inroad to preventing pathogens from inhabiting the host is to impede their contact with the host. Or if this fails, to direct the immune system towards an epitope expressed by the perpetrator in order to clear it rapidly and effectively once it enters the deeper tissue spaces of the body. Traditional vaccine strategies that involve parenteral immunization do often not prevent initial interaction between the pathogen and the host at the mucosal surface. In this way, the pathogen is allowed to establish a beachhead, so to speak, from which it can further explore its potential habitat. Our body has developed similar mechanisms to defend itself against colonization by type 1 fimbriated invaders. These including soluble D-mannosylated glycoproteins in the urine (Tamm-Horsfall protein) and in the mucus which block adhesion to epithelial cells, and immunoglobulins in the gut (secretory IgA) and in the blood, lymphatics and tissue spaces (IgM, IgG). However, due to the high antigenic variation of the predominant FimA subunits among the different strains of *E. coli* expressing type 1 fimbriae, the normal immune response is often not enough. This means, that an acquired immune response directed against FimA of one strain often do not recognize another type 1 fimbriated strain as its FimA molecules are immunologically different. This is illustrated by the fact, that several efforts have been tried, with mixed success, to boost the immune response by immunizing with purified type 1 fimbriae. In one trial (Silverblatt and Cohen, 1979), immunization with purified fimbriae was enough to significantly protect rats against urinary tract infection compared to the unimmunized controls. However, not all attempts to use this method have been successful. Immunization of humans with type 1 fimbriae generated high serum antibody titers against type 1 fimbriae, but did not provide protection against colonization of the intestine by type 1 fimbriated enterotoxigenic *E. coli*, even though high IgG antibody titers were seen (Levine *et al*., 1982). The reason this immunization failed could be because the antibodies did not cross-react with fimbriae of other strains due to the high antigenic variation of type 1 fimbriae (see below). Alternatively, it could be due to immune tolerance or because the route of administration. Systemic administration of antigens does often not result in specific secretory IgA (sIgA) in the gut (or other mucosal surfaces). To best achieve a mucosal sIgA response, administration of antigens should be made directly to the mucosal surfaces. In addition, the existence of a common network within the general mucosal immune system permits immunization on one mucosal surface to induce secretion of antigen-specific sIgA at distal mucosal sites (Walker, 1994).

FimH as a protective agent against infections

Because FimA is the most abundant of the fimbrial proteins, it is also the primary antigenic determinant against which the immune system is most likely to direct its response. This immunological response imposes significant selective pressure on FimA, and thereby the *fimA* gene, and leads to many serologically distinct FimA molecules (Boyd and Hartl., 1998). Creating a vaccine based on FimA, therefore, would likely be

futile, as it would be virtually impossible for it to protect against all the serologic groups.

To circumvent this problem, it may be more sensible to direct the immune system response directly towards the adhesin, FimH. Due to the receptor binding property of FimH, there are limits to what alterations it can accommodate without destroying the receptor recognition ability. This on the other hand does not mean that changes in this protein are impossible, as demonstrated by linker and epitope insertion (se below). However, changes in the receptor domain(s) can probably not be well tolerated, so this is exactly the domain, which would be relevant to use in a vaccine. However, the low relative amounts of FimH in fimbriae makes the immune response against FimH almost undetectable when whole type 1 fimbriae are used as an antigen. Purified FimH elicits a very strong and specific immune response against the adhesin that is capable of preventing type 1 fimbriated *E. coli* from binding to eukaryotic cells (Abraham *et al*., 1988). Even antibodies raised against the first 103 amino acids of the mature FimH fused to a carrier protein (MalE) (Thankavel *et al*., 1997) or against synthetic peptides spanning the mannose binding domain were enough to specifically block type 1 fimbriae-mediated bacterial adhesion to mouse bladder epithelial cells *in situ*. In addition to this, intraperitonealy administered antiserum (IgG) raised against residues 1-25 of FimH prevented mouse bladder colonization of intravesicularly challenged type 1 fimbriated *E. coli*. Immunization with this small peptide also protected mice against urinary tract infections, presumably because of the high IgG and IgA titer in the urine. In a similar study (Langermann *et al*., 1997), these finding were shown to be valid for purified FimH as well. Here, passive systemic administration of immune sera directed against FimH also resulted in reduced colonization by uropathogenic *E. coli*.

Live type 1 fimbrial vaccines

The successful vaccination of mice and the subsequent prevention of colonization by pathogenic *E. coli* described above all required large-scale purification of FimH. Unfortunately, FimH is only made in very small amounts and in the absence of the FimC chaperone it is rapidly degraded by periplasmic proteases (Hultgren *et al*., 1991; Jones *et al*., 1995). Thus, purification of the amounts of FimH that would be required for a large-scale vaccination program has not been feasible, due to degradation and subsequent low yield. It may prove to be more rational to optimize the amount of FimH produced by non-pathogenic *E. coli* and then to either use purified fimbriae or to use intact bacteria directly as live vaccines. Furthermore, such live vaccines could be employed directly at the site of desired immunity or to other mucosal surfaces immunologically connected to this through the mucosal network and thereby only elicit a local immune response. Initial attempts to create such vaccine candidates have proven unsuccessful (Abraham *et al*., 1988; Hasman, unpublished), mostly due to the rapid degradation of excess FimH in the periplasm. Increasing the amount of FimC in the periplasm partly overcame this problem (Jones *et al*., 1995; Hasman *et al*., Chapter 1), but an increase in periplasmic FimH alone does not result in the incorporation of increased amounts of FimH within the fimbriae (Hasman *et al*., Chapter 1). Introducing extra copies of the outer membrane protein FimD did not significantly alter this picture, but adding additional copies of FimF and FimG along with FimC and FimD did result in the production of type 1 fimbriated bacteria containing much more FimH, approximately 25 times the wild-type levels. Furthermore, a 5 to 10 fold increase of the adhesin within the fimbriae was observed. Hopefully, this increase will be sufficient to induce a potent protective immune response against FimH, but this has not been tested yet. As will be seen in the next section, it may also

be possible to utilize type 1 fimbriae carrying increased proportion of the FimH adhesin in other ways, such as in the presentation of other foreign epitopes.

Recombinant fimbrial vaccines displaying foreign epitopes

Due to the characteristics of the fimbrial structural proteins: surface presentation, large numbers of fimbriae per cell and strong antigenicity, it has been suggested that these proteins could act as carriers of foreign epitopes. In this way, the foreign epitopes can be expressed at the surface of the bacteria in high copy number. This type of antigenic presentation could have several advantages: (i) the epitope is located outside the cell, where it can not be attacked by cellular proteases, (ii) the epitopes can be expressed in vast numbers (about 500.000 per cell in the case of FimA), (iii) the epitope is presented on the surface, where it is most prone to encounter the cells of the immune system. Furthermore, various foreign epitopes could be incorporated at the same time by employing different carrier proteins or multiple fimbrial systems. That this inroad is possible has been shown by Klemm and coworkers (Hedegaard and Klemm, 1989; Pallesen *et al*., 1995; Stentebjerg-Olesen *et al*., 1997). By examining the hydrophilicity profile and predicted secondary structure of FimA, several candidate positions were chosen and small epitopes from foot-and-mouth disease virus (FMDV), hepatitis B surface antigen (HbsAg) and the C3 epitope from the VP1 capsid protein of type 1 poliovirus were inserted into these sites. It was possible to detect all of these epitopes, as well as fimbriae production, by using specific antibodies against each of these in Western blots, thereby proving the feasibility of the method (Hedegaard and Klemm, 1989). These tests have been further expanded to include an epitope from the cholera toxin B chain (CTB) and this epitope was shown to be present on the surface of the bacteria by immunofluorescence microscopy with antibodies directed against the CTB (Stentebjerg-Olesen *et al*., 1997). Not only can the FimA protein be used as a carrier, but also FimH has been shown to accommodate foreign peptides and to present these on the bacterial surface without destroying the mannose-binding ability of the fimbriae (Pallesen *et al*., 1995). In this study, a fairly large peptide of 52 amino acids containing the complete PreS2 fragment of the hepatitis B surface antigen and a another smaller (15 amino acids) epitope from CTB were both shown to be presented on the surface and to be recognized by specific antisera. As has been mentioned above, because the natural content of FimH within the fimbriae is low compared to FimA, using FimH to construct a vaccine against one of these foreign epitopes might not be efficient without increasing the proportion of FimH within the fimbriae. One of the subjects of this thesis is the genetic manipulation of the *fim* gene cluster to enhance production of fimbriae containing increased amounts of FimH. From the experiments presented above, it therefore seems reasonable that these types of procedures should increase the feasibility of using FimH or chimeric FimH proteins in vaccine trials.

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PART II

Antigen 43 and the auto-transporter family

Another phase variable surface component of *E. coli* is the outer membrane protein complex antigen 43 (Ag43), the product of the *flu* gene first described by Diderichsen (1980). It confers auto-aggregation of *E. coli* K-12 strains and additionally seems to play a role in colony morphology development. Ag43 belongs to a family of large outer membrane protein complexes called the autotransporters. Relatively little attention has been given to this protein complex up until now and therefore the data available is limited. It does, however, show similarity to some of the other autotransporters. Therefore, this family of proteins will be described with emphasis on the common features of the autotransporters and two of the members, *viz.* AIDA-I and Ag43.

Autotransporters

The autotransporter class of bacterial surface proteins (reviewed by Henderson *et al*., 1998) consists of a heterologous group of large outer membrane proteins (sizes range between 800 and 1600 amino acids). They are found among a broad variety of bacterial species including *E. coli*, *Nesseria gonorrhoeae* and *Haemophilus influenzae*. Autotransporter proteins normally consist of three distinct functional domains: (i) a signal peptide required to cross the inner membrane, (ii) a passenger domain (often called the α fragment), which contains the active/reactive domain, and (iii) the transmembrane domain (the β fragment), which serves as the anchor and translocator for the α fragment (Figure II.1).

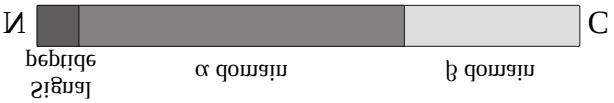


figure II.1. Graphical representation of protein chain of the autotransporters. N- and C-terminal ends are shown (see text for details).

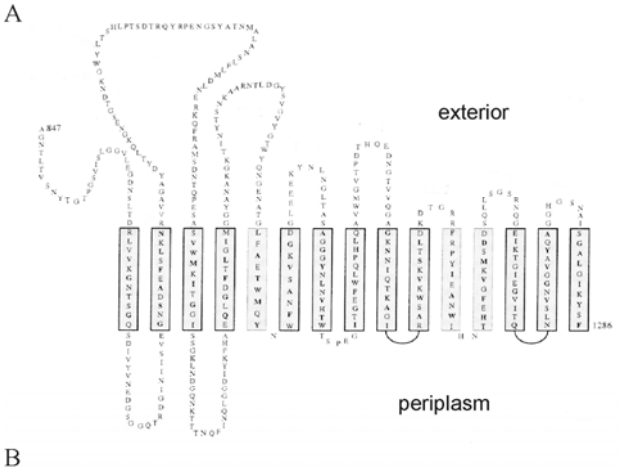
The signal peptides of the autotransporters are often remarkably long (approximately 50 amino acids). Nevertheless, they all contain the features required to cross the inner membrane in a *sec* dependant manner: an N-terminus of positively charged amino acids, a hydrophobic region composed of neutral amino acids and a C domain containing a consensus sequence for the signal peptidase (Henderson *et al*., 1998). This suggests, that the autotransporters in general utilize the *sec* system of the inner membrane to gain access to the periplasm and the signal peptide is cleaved off by the signal peptidase (reviewed by Pugsley, 1993).

The α domains are the most variable part of the proteins. It is these domains, which contain the active part of the autotransporters and are as such the most interesting part of the protein, where the signal peptide and the β domain merely serve as tools to present the α domain on the surface. The size of the α domain can

Table 1. The autotransporters ordered by function. Adapted from Henderson *et al*. (1998).

Unknown function	Adhesins	Proteases	Toxins	Invasins	Mediaters of motility	Serum resistance
EspC	AIDA-I	Ssp	VacA	Hap	IcsA	BrkA
SepA	Rsh	Ssp-h1	Pet	-	-	-
Hia	TcfA	Ssp-h2	-	-	-	-
rOmpA	Ag43	EspP	-	-	-	-
rOmpB	Hap	PssA	-	-	-	-
SlpT	Hsf	IgA1 proteases	-	-	-	-
Hsr	Pertactins	-	-	-	-	-
-	UspA1	-	-	-	-	-

A



B

[SATQRGHF]-[AKLTQNFGLIV]-[FGATLISN]-[GPDSNKIATW]-[KRHNDQS]-X-X-X-[DPAQHKG]-[QNDWKYFITLGH]-[ASTQNG]-[LIVFGASY]-[NQHEGAIFTS]-[LIVA]-[NGSKT]-[LIVMFYG]-[RKQSTGN]-[LIFVYW]-[STAKRNVQEM]-[FW]

figure II.2. (A) Proposed topology of AIDA-I^c in the outer membrane (from Suhr *et al*., 1996). According to the secondary-structure prediction AIDA-I^c is composed 15 amphipatic β-strands with 9-13 amino acid residues per strand. (B) Signature sequence of the C-terminal of the autotransporters. Each bracket indicate one amino acid position. X's represent any amino acid (adapted from Henderson *et al*., 1998)

vary considerably depending on its function, and α domains of 400 to 700 amino acids are not unusual. The function of the α domains can be very diverse, spanning from proteases to toxins and adhesins (Table 1). The common theme of many of these is their function, which seems to be connected to virulence of the carrier organism. However, such a relation has not yet been established between Ag43 and *E. coli*.

The β domains are the transmembrane part of the proteins. They contain from 10 to 18 amphipathic β-sheets, each consisting of about 10 amino acids, and have the ability to transverse the outer membrane. The β domain thus serves as an anchor for the passenger domain; it may also function as a pore through which the α domain is channeled in order to gain access to the surface (Pugsley, 1993; Henderson *et al*., 1998). Due to the sequence homology of part of the β domain to some serine proteases, it is believed that, at least, some of the β domains contain protease activity. This is further fueled by the fact that some of the autotransporters are cleaved between the α and the β domain independently of several of the known proteases located in the outer membrane (Suhr *et al*., 1996; Henderson and Owen, 1999), and therefore it may possess autocatalytic activity. However, conclusive evidence for this tenet is not available and at least one other autotransporter, AIDA-I, requires an auxiliary protein in order to be processed correctly (Benz and Schmidt, 1992a).

Apart from the above-mentioned characteristics, the autotransporters have several features in common. The ones that possess an unusually long signal peptide carry an eight-amino acid motif (IAVSELAR), which varies only slightly within the group (Henderson *et al*., 1998). Also, cysteine residues are often absent in the mature autotransporters. This is most likely to avoid the formation of cysteine bridges, which would impede autotransport across the outer membrane (Jose *et al*., 1996). Finally, they also have a rather broad, C-terminal signature sequence of the β fragment (figure II.2B). This sequence is believed to be involved in the attachment and integration of the β domain to the outer membrane as deletion of, or certain mutations in, this extreme C terminal part have been shown to reduce the localization of at least one of the auto-transporters (Hendrixson *et al*., 1997).

The secretion mechanism of the autotransporters

The autotransporters utilize a common, unique, type of mechanism in order to get from the cytosol to the outer membrane. As their name implies, they seem to contain all the information necessary to ensure the translocation. The precise mechanism is not understood completely at this point, but a model (figure II.3) has been proposed (Henderson *et al*., 1998). Once the signal peptide has promoted the transport across the

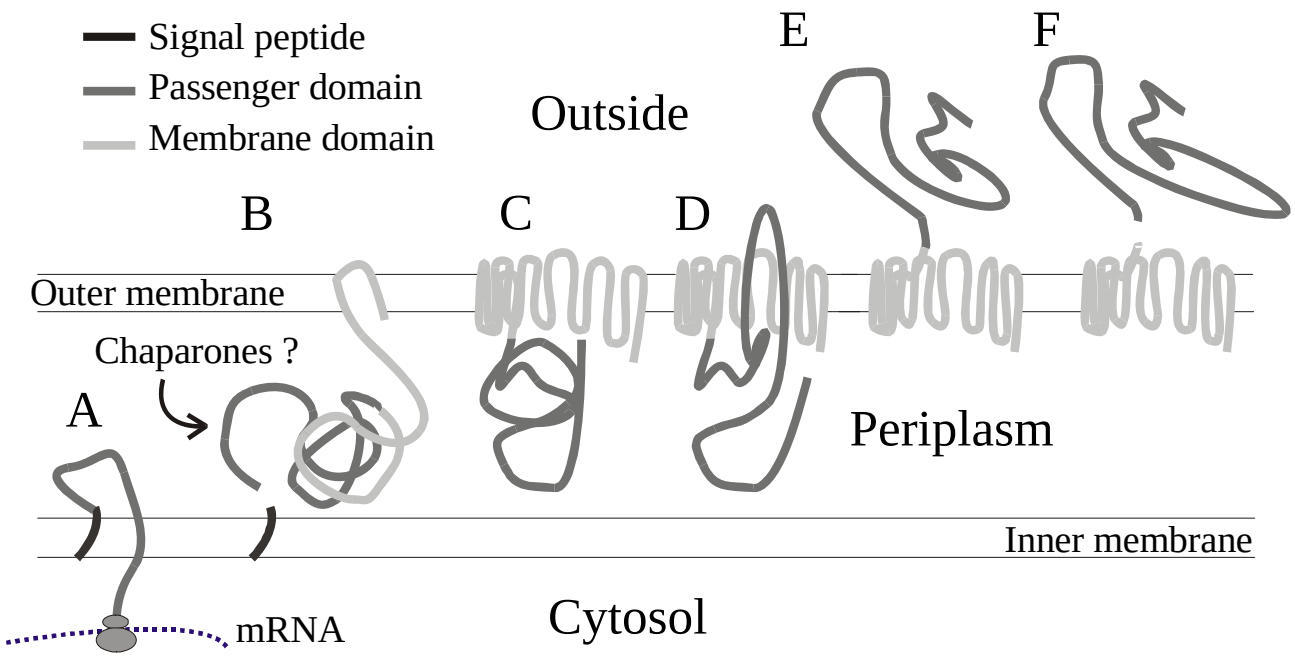


figure II.3. Model (out of scale) of the autotransporter biogenesis. The signal peptide (black) enables the autotransporter to reach the periplasm (A), where it, presumably, connects with periplasmic chaperones (B). Then the membrane (β) domain (light gray) inserts in the outer membrane and form a pore (C) through which the passenger (α) domain (dark gray) is subsequently pulled (D). Once the α domain is outside et can either remain associated with the β domain (E) or, after proteolytic cleavage, detach from the cell (F).

inner membrane (figure II.3A), the protein enters the periplasm. The precise fate of the autotransporter in the periplasm is not known, but it is assumed, that either it connects with periplasmic chaperones or that the protein chain contains autochaperone properties, which protects it from degradation. In support of the former is that one of these autotransporters (*orfB* or AIDA-I) appears to require a secondary protein (the *orfA* gene product) in order to be correctly processed (Figure II.3B). When the autotransporter reaches the outer membrane, the extreme C terminal part of the chain inserts into to membrane layer and pulls the β fragment along with it (figure II.3C). The amphipathic β-sheets of the β-fragment serve as transmembrane regions ordered in an anti-parallel barrel structure. It is through this barrel structure that the α domain is assumed to be pulled and subsequently translocated to the surface of the bacterium (figure II.3D). Once the α domain has reached the surface, either one or two things can happen depending on the function of the passenger domain (figure II.3E). Either the α and the β domain remain covalently bound through the peptide chain, or the peptide chain is proteolytically cleaved at the junction between the α and β domains (figure II.3F). If the latter occurs, the two domains can either part whereby the α domain is released to the exterior, as seen with the VirG protein (Goldberg *et al.*, 1993; Fukuda *et al.*, 1995) and the IgA proteases (Kornfeld and Plaut, 1981; Klauser *et al.*, 1993) or they can remain together through non-covalent binding as seen with AIDA-I (Suhr *et al.*, 1996) and Ag43 (Owen *et al.*, 1987).

AIDA-I

Adherence phenotypes of Enteropathogenic E. coli

Enteropathogenic *E. coli* (EPEC) are the major bacterial cause of neonatal and infantile

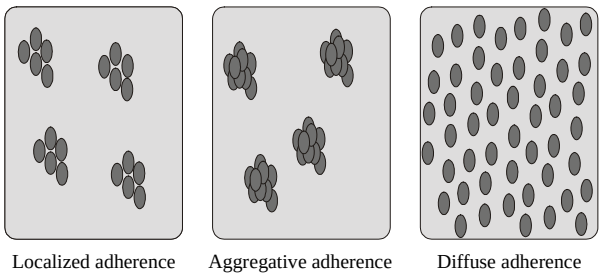


figure II.4. Model of the three different of adherence observed by Enteropathogenic *E. coli*: Localized adherence, Aggregative adherence and diffuse adherence.

gastroenteritis in humans (Levine, 1987). They do not have the ability to invade the epithelium, but as they adhere to the epithelial cells, they cause severe diarrhea. EPEC strains possess several different means of attaching to epithelial cells. These are divided into three distinct groups (figure II.4): localized adherence in which the bacteria adhere in discrete microcolonies, aggregative adherence (reviewed by Law and Chart, 1998), whereby aggregated bacteria attach to cells, and diffuse adherence, where the bacteria adhere evenly to the surface of epithelial cells (Benz and Schmidt, 1989). The localized adherence is connected to the EAF plasmid, which contains the gene cluster for bundle forming pili (Girón *et al.*, 1993) and to a chromosomally located gene cluster termed *eae* (for *E. coli* attaching and effacing). EPEC strains carrying these two factors are able to adhere to brush borders and gain intimate contact to the epithelial cells through the *eaeA* gene product (intemin), a 94 kDa outer membrane protein (Jerse and Kaper, 1991; Donnenberg and Kaper, 1992). Aggregative adherent EPEC also employ fimbriae as a mean of attachment. These fimbriae, called aggregative adherence fimbriae (AAF/I and AAF/II) are also plasmid encoded along with several toxins such as the EAggEC heat-stable enterotoxin, EAST1 (Nataro *et al.*, 1993) and the autotransporter Plasmid encoded toxin, Pet (Eslava *et al.*, 1998). Finally, diffuse adherence of EPEC is caused by one of two different mechanisms, as both F1845 fimbriae (Bilge *et al.*, 1989) and the outer membrane protein AIDA-I have been shown to be involved in diffuse adherence (Benz and Schmidt, 1989).

AIDA-I

The plasmid-encoded surface protein AIDA-I (Benz and Schmidt, 1989) also belongs to the autotransporter family of proteins. It shows 48% similarity and 25% identity with the VirG protein, which is connected to intercellular spreading of the invasive *Shigella flexneri* bacteria (Benz and Schmidt, 1992a; Suzuki *et al.*, 1995). The AIDA-I precursor is a classical autotransporter composed of a 49 amino acid signal peptide, a passenger domain (AIDA-I) and a transmembrane domain (AIDA-I^C). It is synthesized as a preprotein, from a 3.9 kb open reading frame (*orfB*), with the size of 132 kDa which is first cleaved when it crosses the inner membrane (to loose the signal peptide) and further processed between the α and β

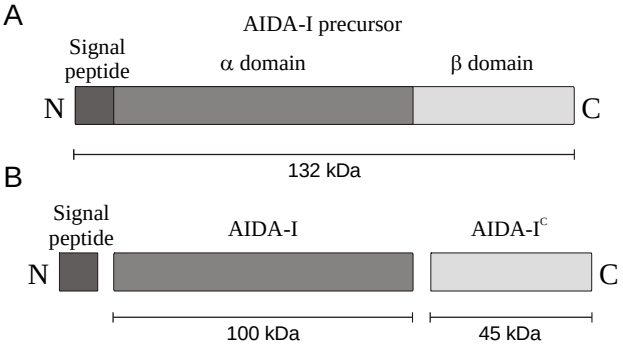
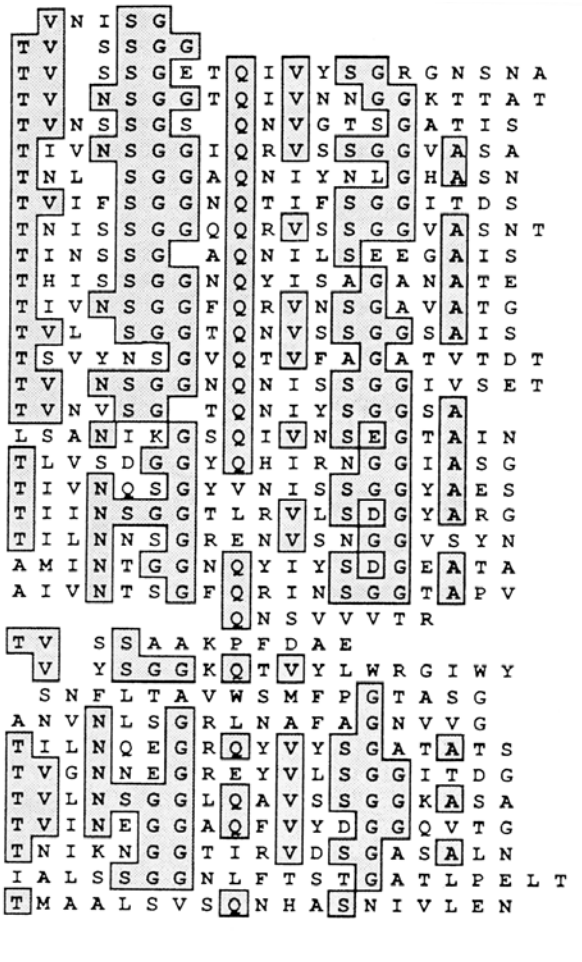


figure II.5. (A) The AIDA-I precursor with the apperent molecular weight indicated below. (B) After two rounds of cleavage, the three shown subunits appear, dividing the precursor into the signal peptide, AIDA-I and AIDA-I^C. Apperent sizes are indicated below.

domains when it reaches the outer membrane. This results in two peptides (figure II.5) of apparent molecular sizes of 100 kDa (AIDA-I) and 45 kDa (AIDA-I^C) (Benz and Schmidt, 1989; Suhr *et al.*, 1996). The correct processing is further dependent on the product of an upstream gene (*orfA*), which codes for a 45 kDa protein. If this is not expressed along

with the *orfB* product, the latter can not be processed correctly and the diffuse adherence phenotype is lost (Benz and Schmidt, 1992a). Whether this protein performs a protective role in the outer membrane against membrane located proteases or as a chaperone in the periplasm is not known (Suhr *et al*., 1996).

It was shown (Suhr *et al*., 1996), that the passenger domain is not required to incorporate the β fragment ($AIDA^C$) within the outer membrane, as chimeric fusion proteins comprised of a signal peptide from the *pelB* gene and the β fragment of AIDA-I (including the cleavage site) were detected on the bacterial surface. However, apart from cleavage of the signal peptide, this chimeric protein was not processed to give the mature $AIDA^C$, indicating that the α fragment participates in the cleavage process. Several of the outer membrane located proteases have been examined for their possible role in the cleavage process. However, both DegP, OmpP and OmpT protease-deficient *E. coli* strains as well as avirulent salmonellae and shigella were able to present AIDA-I on the surface, further indicating an autocatalytic cleavage process of this autotransporter (Suhr *et al*., 1996).



T V . N S G G . Q . V . S G G . A . .

figure II.6. Amino acid sequence of the repeat region of AIDA-I (From Benz and Schmidt, 1992). The consensus sequence is shown on the bottom of the figure.

AIDA-I promotes adherence to epithelia cells

Purified AIDA-I coated to micro titer plates has been shown to mediate adherence of HeLa cells, which could be inhibited by both antisera and F_{ab} fragments directed against AIDA-I when incubated with these prior to the addition of the HeLa cells (Benz and Schmidt, 1992b). Even though this clearly shows that AIDA-I is responsible for the adhesion, neither the receptor nor the receptor-binding domain of AIDA-I has yet been determined. Often, an Arg-Gly-Asp (RGD) motif is used by adhesins in order to bind to receptors (D'Suoza *et al*., 1991). This has shown to be the case with the autotransporter and adhesin Pertactin from *Bordetella pertussis*, which causes whooping cough in humans (Leininger *et al*., 1991). However AIDA-I does not contain such a RGD motif indicating a different mode of attachment, which strongly suggest another mechanism of adherence.

Apart from the common motifs shared by many of the autotransporters listed above, AIDA-I also contains a large number of repetitive sequences within the passenger fragment. A stretch of 21 (incomplete) repeats consisting of 19-20 amino acids each, is located in the N terminal part of the protein (figure 4.6). This is followed by a second stretch of 10 repeats with a less significant fit to the consensus sequence: TVXNSGGXQXVXSGGXAXX (where X represents any amino acid) (Benz and Schmidt, 1992a). These repeats have been suggested to function in receptor recognition, a

feature also seen with other repeat sequence domains (Felmlee and Welch, 1988).

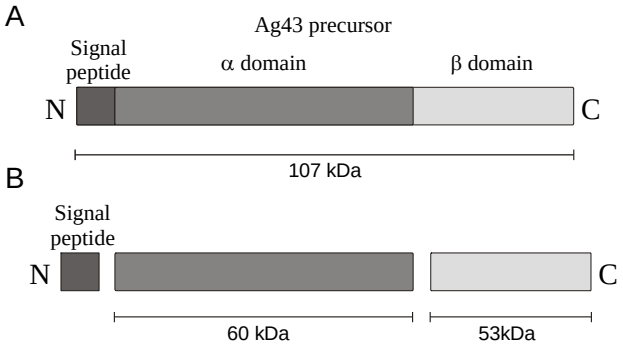


figure II.7. (A)Graphical representation of the Antigen 43 precursor and (B) the resulting fragments after translocation across the outer membrane. The apparent molecular sizes, as determined by Caffrey *et al*. (1989) are indicated below.

Antigen 43

Antigen 43, the fluffing protein from E. coli K-12

Antigen 43 (Ag43) is an autotransporter protein with some homology to AIDA-I (23% identity and 70% similarity overall and 31% identity and 72% similarity within the α domains) (Owen *et al*., 1996). It was first detected in 1983 as one (number 43) of the *E. coli* surface antigens (Owen, 1983; Owen *et al*., 1987). It was found to consist of an α and β subunit in equal amounts on the cell surface. Furthermore, the α subunit was shown to non-covalently attached to the β subunit, as the former could be detached from the surface by mild heat treatment (60 °C for 20 minutes) whereas the latter could not. The α and the β subunit had an apparent size of 60 and 53 kDa, respectively (figure II.7). Notably, the size of the β subunit changes to an apparent size of 37 kDa if only heated to 70 °C or below before SDS-PAGE (Owen *et al*., 1987). This is often seen with membrane proteins indicating that this subunit is the membrane-integrated part. Very recently, the topology of the β-fragment has been suggested to be composed of 18 anti-parallel amphipathic β strands (figure II.8), each containing from 11 to 17 amino acids, but this is entirely based on empiric calculations and is thus purely speculative (Henderson and Owen, 1999). Ag43 has been shown to be the product of the *flu* gene (see later), located at 43 minutes on the *E. coli* K-12 chromosome

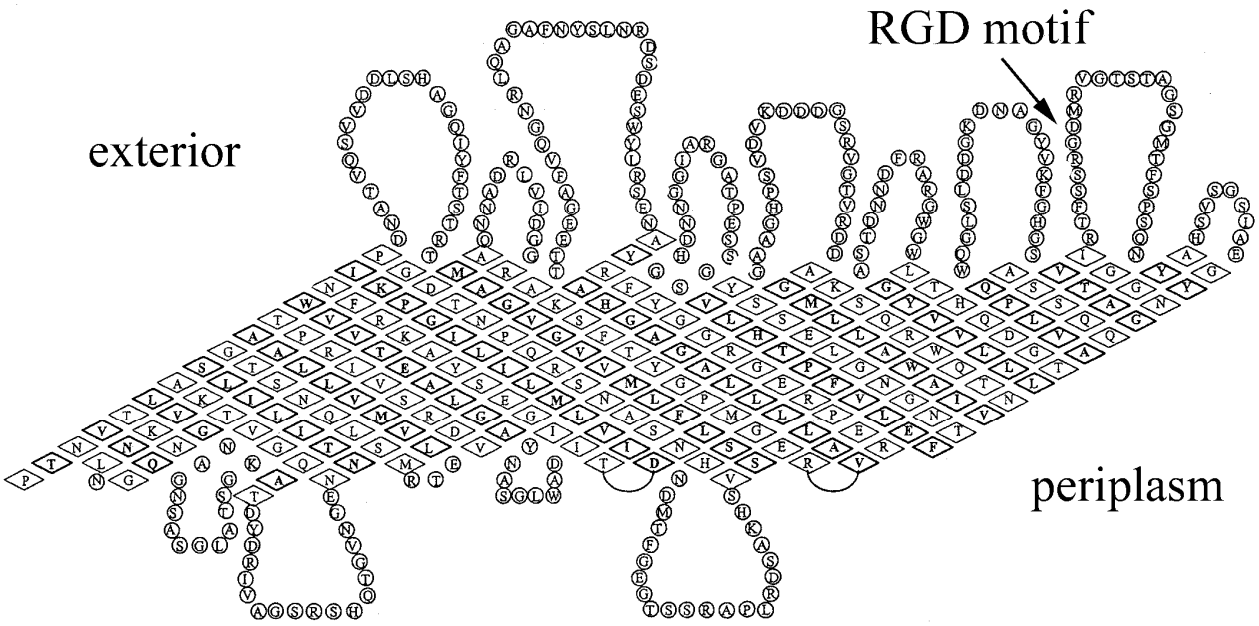


figure II.8. Proposed topology of Antigen 43 in the outer membrane (From Henderson and Owen, 1999). According to the secondary-structure prediction Antigen 43 is composed 18 amphipatic β-strands with 11-17 amino acid residues per strand. The RGD sequence is indicated by a black arrow.

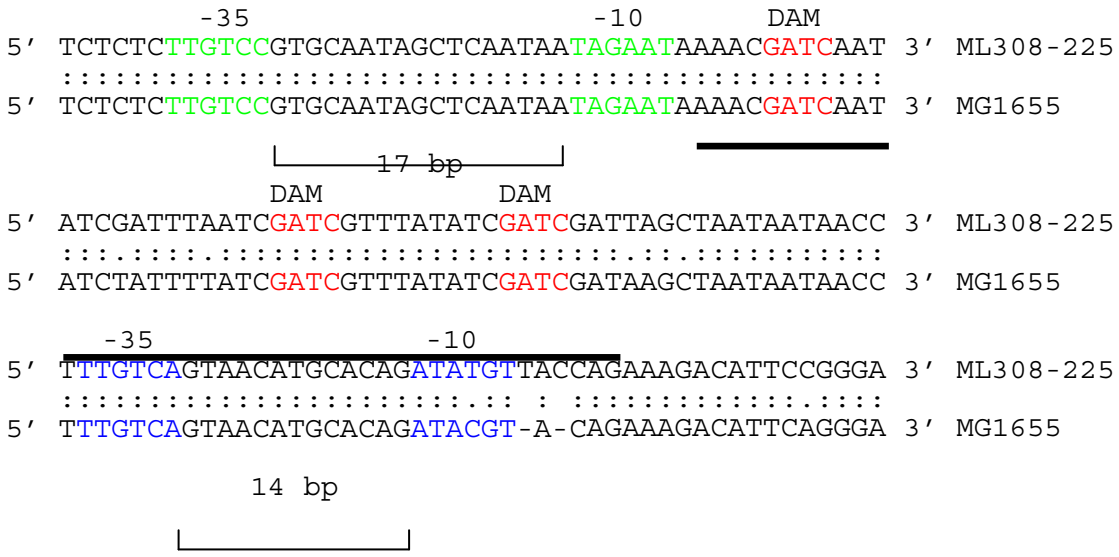


figure II.9. The promoter region of *E. coli* ML308-225 (upper strand) and *E. coli* K-12 MG1655 (Lower strand). Suggested promoters are shown in green (Hasman *et al.* Chapter 2) and blue (Henderson and Owen, 1999) with their respective spacer indicated below. Thick black time indicates the proposed OxyR motif (Henderson and Owen, 1999), Dam sites are given in red.

(Diderichsen, 1980; Henderson *et al.*, 1997a), thereby linking its name to its location in a completely coincidental manner.

The *flu* gene of *E. coli* K-12 strain MG1655 encompass at least two overlapping open reading frames, one of 1091 and one of 1039 amino acids. N-terminal sequencing (Caffrey and Owen, 1989) of the mature α subunit revealed that the cleavage site between the signal peptide and the α subunit was after the 102nd or after the 52nd amino acid, depending on the reading frame chosen. This suggests the smallest of the reading frames to be the correct one, as this leads to a signal peptide with the size of 52 amino acids, which is in good agreement with the sizes of signal peptides of other autotransporters (see above). Furthermore, this open reading frame has a fairly good ribosome binding site (AGGAA) in front of it, which further confirms this reading frame to be the correct (Hasman *et al.*, Chapter 2). A putative σ^{70} promoter located approximately 230 bp upstream of the *flu* gene (Figure II.9) has been suggested to control expression of Ag43 (Hasman *et al.*, Chapter 2). The *flu* genes from *E. coli* K-12 and the recently described *E. coli* strain ML308-225 (serotype O13:068:H$^-$) show 98% identity at the nucleotide level. However, minor differences exist, especially in the promoter region suggested for the ML308-225 strain (Henderson and Owen, 1999), but not in the promoter region suggested for the K-12 strain (Hasman *et al.*, Chapter 2). N-terminal sequencing of the β subunit suggests the cleavage point between the α and the β subunit (Owen *et al.*, 1996) to be after the 499th amino acid of the mature protein (Henderson and Owen, 1999; Hasman *et al.*, Chapter 2) and the expression level of Ag43 has been estimated to approximately 50,000 proteins/cell (Owen *et al.*, 1996).

Ag43 promotes auto-aggregation of liquid cultures

It was also noted (Caffrey and Owen, 1989) that the yield of Ag43 varied significantly from batch to batch of the same cells under the

same conditions. An explanation for this was not found until the amino acid sequence of the N terminal end of the α subunit was determined and a 1.2 kbp fragment of the gene was synthesized by PCR and compared to the Kohara gene bank (Owen *et al*., 1996; Henderson *et al*., 1997a). By this method it was shown, that the PCR product hybridized to two overlapping clones covering the 43rd minute of the *E. coli* chromosome. This region was previous shown to encompass the *flu* gene (Diderichsen, 1980), which was involved in auto-aggregation (fluffing) of certain *E. coli* K-12 strains. Auto-aggregating cells spontaneously form aggregates when left in static conditions and precipitate to the bottom of the tube within few hours. The product of the *flu* gene was shown to be phase varying, oscillating between a fluffing and a non-fluffing phenotype (Diderichsen, 1980). Subsequent Western blotting and immunofluorescence microscopy with antibodies directed against Ag43 underlined the correlation between the *flu* gene product and Ag43 (Henderson *et al*., 1997a). Though Diderichsen imagined a regulatory role for the *flu* gene product and by default assigned the *flu* gene product to the non-fluffing phenotype (*flu*$^+$) and the fluffing phenotype to the lack of gene expression (*flu*), it was obvious from the later experiments that the opposite relation was actually the case (Owen *et al*., 1996). That is, the Ag43 protein was the product of the *flu* gene and responsible for the auto-aggregating phenotype.

Diderichsen (1980) first described the three classical strains used in the present study of Ag43. Two of these, BD1511 and BD1512, are in fact the same strain, which only differ in their *flu* status. BD1511 is non-fluffing (Ag43$^-$) and BD1512 is fluffing (Ag43$^+$), but due to the metastable nature of the *flu* gene product, these two strains constantly switch phenotype with a frequency of approximately 2.2×10^{-3} from Ag43$^+$ to Ag43$^-$ and 1.0×10^{-3} from Ag43$^-$ to Ag43$^+$ (Owen *et al*., 1996). This stochastic phase switching phenomenon makes it difficult to carry out controlled experiments, as a fairly large portion of e.g. BD1511 cells grown over night will have changed to the opposite phenotype and vice versa. A strain, where the phase is locked in the Ag43$^+$ phenotype also exists (BD1302), but this strain is not isogenic with the others (BD1511/BD1512) and therefore not directly comparable with these. Furthermore, the locking of the *flu* gene is a result of a larger deletion of the chromosome (see below). Therefore, the lack of defined mutants has severely hampered investigation of the auto-aggregation phenomenon in more detail.

Ag43 is the only component involved in auto-aggregation

To examine, if there were other (surface) components involved in the auto-aggregation phenotype, a defined *flu* mutant was constructed by inserting a *tet* cassette into the chromosomally located *flu* gene (Hasman *et al*., Chapter 2). The construct was made in such a way, that transcription of eventual downstream genes was not affected by the insertion. This mutant ceased to exhibit the auto-aggregation phenotype and re-introducing the *flu* gene *in trans* on a plasmid restored the parent phenotype, thereby proving that the *flu* gene product was directly responsible for this

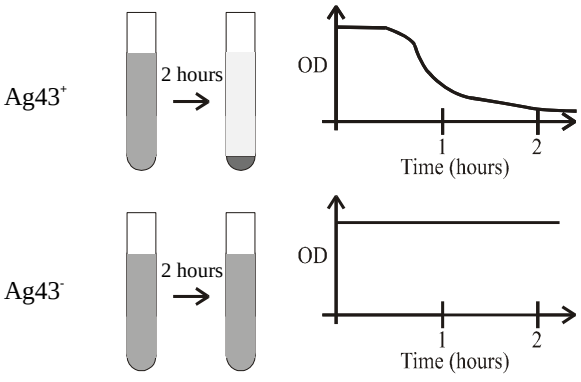


figure II.10. Illustration of the auto-aggregation phenomenon (top) compared to a bacterial culture, where the cell are not expressing Antigen 43 (bottom). Representative sedimentation curves are given to the right.

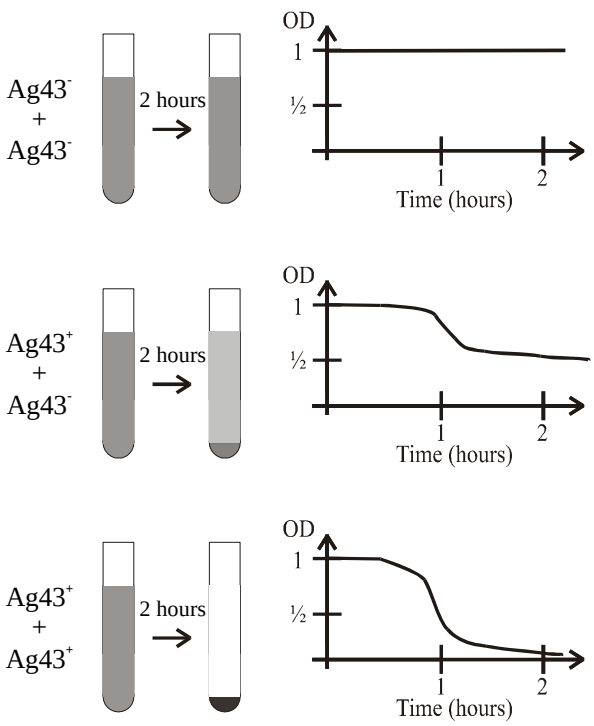


figure II.11. The effect of mixing either (top) an $Ag43^-$ with an $Ag43^-$ strain, (Middle) an $Ag43^-$ with an $Ag43^+$ strain or (bottom) an $Ag43^+$ with an Ag43+ strain.

distinct phenotype (figure II.10). Mixing the parent with the *flu* mutant in equal amounts showed, that auto-aggregation required the presence of Ag43 on both cells, as only the parent precipitated out and left the mutant remaining in suspension (figure II.11). The amino acids involved in this Ag43-Ag43 recognition are not yet characterized. Ag43 contains two RGD (Arg-Gly-Asp) sequences which could be responsible for the binding (see above). As both the α and the β fragment contain RGD sequences, these could also be involved in the non-covalent association of the two fragments. Both possibilities are supported of the fact that the proposed topology of the β fragment presents the RGD sequence within an extracellular loop, which has the (theoretical) potential to interact with α fragment (figure II.8).

Ag43 is involved in colony morphology development

Another striking difference between BD1511 ($Ag43^-$) and BD1512 ($Ag43^+$) is that the former gives rise to small, high, smooth looking colonies (termed "glossy") on agar plates and the latter creates large, flat, rough looking colonies (termed "frizzy"). These two different colony morphologies have been termed "Form 2" and "Form 1", respectively (Warne *et al.*, 1990). Due to the fact that cells of Form 2 colonies express Ag43 and Form 1 does not, it has been suggested that Ag43 is directly responsible for the differences between the two forms. This, however seems not to be the complete story. A *flu* mutant of BD1511/BD1512 still undergoes phase variation between a small and large colony morphology as seen with the isogenic parents, but the frizzy surface phenotype of the large colonies are no longer seen (Hasman, Chapter 3). Actually, this new colony morphology (termed "Form 3"), where the colonies are large, flat and have a smooth surface, is also seen when cells originating from a Form 1 colony are converted to Form 2 (and vice versa), indicating that the transformation between the two occurs through a, third, transition state. When these Form 3 colonies originating from either Form 1 or Form 2 are picked directly from the plates (thereby omitting growth in liquid suspension over night) and examined by immunofluorescence microscopy, it is evident that they do not express Ag43. Therefore, it can be concluded that Ag43 does not play any role in determining colony size but it is responsible for the frizzy surface appearance of the Form 1 colonies (Hasman, Chapter 3).

Ag43 is regulated by OxyR and dam methylation

As mentioned above, a large deletion phase-locked the BD1302 strain in the $Ag43^+$ state. The deletion, however, was located at a completely different position (at the 89. minute) on the chromosome than where the *flu*

gene was mapped (Diderichsen, 1980). This deletion included the *mor* (*oxyR*) gene (Warne *et al*., 1990), coding for a protein called Mor (OxyR), which regulates several proteins involved in the oxidative stress response (Stortz *et al*., 1990; Mukhopadhyay and Schellhorn, 1997). By constructing a plasmid-born gene library from the chromosome of BD1512, transforming the resulting plasmids into BD1302 and searching for clones that were no longer auto-aggregating, it was shown, that Mor was able to prevent auto-aggregation in BD1302 (Warne *et al*., 1990). However, these authors did not determine if this was as a result of decreased expression of Ag43. This has subsequently been shown in later experiments (Henderson and Owen, 1999; Hasman *et al*., Chapter 2). OxyR exists both in an oxidized and a reduced form. Both forms have been shown to repress gene expression *in vitro*, but only the oxidized form is able to activate transcription (Storz *et al*., 1990; Toledano *et al*., 1994). Expression of Ag43 has been shown to be dependent on the OxyR form, as BD1302 cells carrying the OxyR protein locked in the oxidized form were unable to repress transcription of the *flu* gene, whereas locking OxyR in the reduced form does repress Ag43 expression (Henderson and Owen, 1999).

Introducing a mutation in the deoxyadenosine methylase gene (*dam*), which makes the cell unable to methylate GATC sequences of DNA, completely abolished the ability of wild type strains to express Ag43. However, mutating the *dam* gene in BD1302 did not influence its capability to express Ag43. This led Henderson *et al*. (1997b) to suggest a model (shown in figure II.12), where both OxyR and Dam compete for the promoter region (operator) upstream of the *flu* gene. If the operator is not methylated, OxyR can bind and transcription is repressed but if Dam is able to methylate the operator, then OxyR is no longer able to bind and the RNA polymerase can carry out transcription (Henderson and Owen, 1999). Examination of the upstream region of the *flu* gene of the *E. coli* K-12 strain MG1655 reveals a putative promoter that is different from the one recently suggested by Henderson and Owen (1999), followed by three Dam methylation sites and a possible OxyR binding motif (figure II.9).

Ag43 and type 1 fimbriae

It was previously observed, that glossy colonies contained type 1 fimbriated bacteria whereas frizzy colonies did not (Diderichsen, 1980). This led to the suggestion that the *flu* gene controlled type 1 fimbriation. Recent examination of this possible correlation has not revealed any proof of such a connection

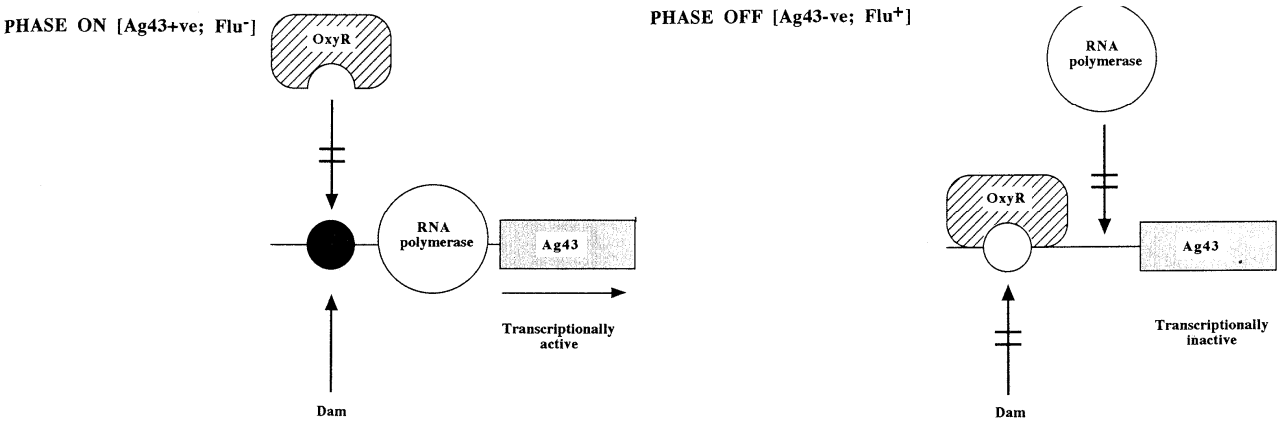


figure II.12. The proposed model to account for the observations obtained with OxyR and Dam (From Henderson *et al*., 1997a). If the Dam methylase acts on the operator before OxyR, the its binding id prohibited, which enable the RNA polymerase to transcribe the *flu* gene (left). On the other hand, if OxyR binds to the operator, Dam and the RNA polymerese are obstructed and on transcription occurs.

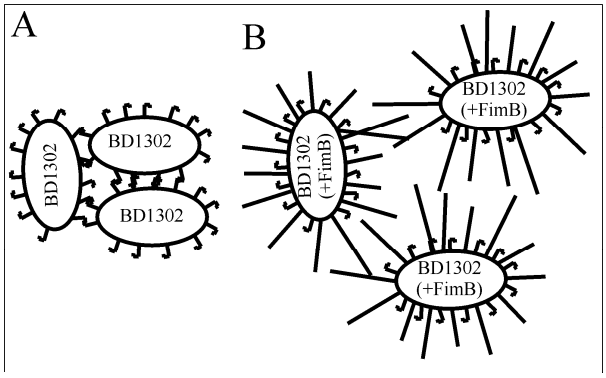


figure II.13. Model illustrating (A) auto-aggregating cells and (B) the effect of fimbriation (black bars), which causes that auto-aggregation to cease, despite the continuous Ag43 production (black hooks).

(Hasman *et al*., Chapter 2). Type 1 fimbriation does, however, influence the Ag43 phenotype, as was illustrated when BD1302 was forced to produce type 1 fimbriae by overproducing the regulatory gene FimB. This resulted in total loss of the auto-aggregation phenotype normally seen with this strain. Immunofluorescence microscopy with specific antibodies directed against Ag43 of strains forced to produce type 1 fimbriae revealed that the loss of auto-aggregation was not as a result of downregulation of Ag43, because the cells expressed the protein on the surface. Furthermore, the same effect could be seen with plasmids carrying either the *fim* genes downstream of an artificial *lac* promoter or the genes of the P fimbriae and the F1C fimbriae. As these fimbrial gene clusters employ completely different regulation mechanisms (Blyn *et al*., 1990; Braaten *et al*., 1994) it seems more likely that the loss of auto-aggregation was caused by the physical presence of fimbriae on the surface (figure II.13), which made Ag43-Ag43 contact impossible (Hasman *et al*., Chapter 2).

This, however, does not explain the phase varying phenotypes of BD1511 and BD1512, where only the former was observed to produce type 1 fimbriae by electron microscopy (Diderichsen, 1980). This relationship was confirmed by employing immunofluorescence microscopy of both Form 1 and Form 2 colonies (Hasman, Chapter 3). Close examination of the *fim* gene cluster of BD1511/BD1512 revealed that one of the type 1 fimbrial regulatory proteins (FimE) was knocked out by an IS*1* insertion element. This is strikingly similar to a previous report connecting colony morphology and a *fimE* mutant (Blomfield *et al*., 1991). Here, the *E. coli* K-12 strain CSH50 was shown to display a phase varying morphotype of small and large colonies similar to the one reported by Diderichsen (1980). The phenotype could be transferred to the non-phase varying strain MG1655 both by allelic complementation as well as by introducing an amber mutation in the *fimE* gene. Reintroducing the *fimE* gene on the chromosome of the BD1511/BD1512 strain set by allelic complementation (Hasman, Chapter 3) resulted in both Form 1 and Form 3 colonies (large), but not Form 2 colonies (small). An equally small fraction of cells from both of these forms expressed type 1 fimbriae, thus abolishing the connection between phase variation of type 1 fimbriae and colony morphology in a wild type situation. The same pattern in colony morphology variation was seen when the complete *fim* gene cluster was deleted from the chromosome of the BD1511/BD1512 strain set. Therefore, Form 2 colonies are only found when the *fim* gene cluster is residing on the chromosome but with the *fimE* gene knocked out. This suggests that FimE neutralize the influence of FimB on solid media. This is properly due to the high switching frequency (0.75/cell/generation) towards the non-fimbriated phenotype, which FimE mediates, compared to low switching frequency (10^{-3}/cell/generation) of FimB (Gally *et al*., 1993).

Ag43 homologues on the E. coli chromosome

A repeat sequence domain (figure II.14) similar to the one found in AIDA-I has been suggested recently for the Ag43 originating from the ML308-225 strain (Henderson and Owen, 1999). Analysis of the Ag43 sequence of *E. coli* K-12 reveals that this protein also

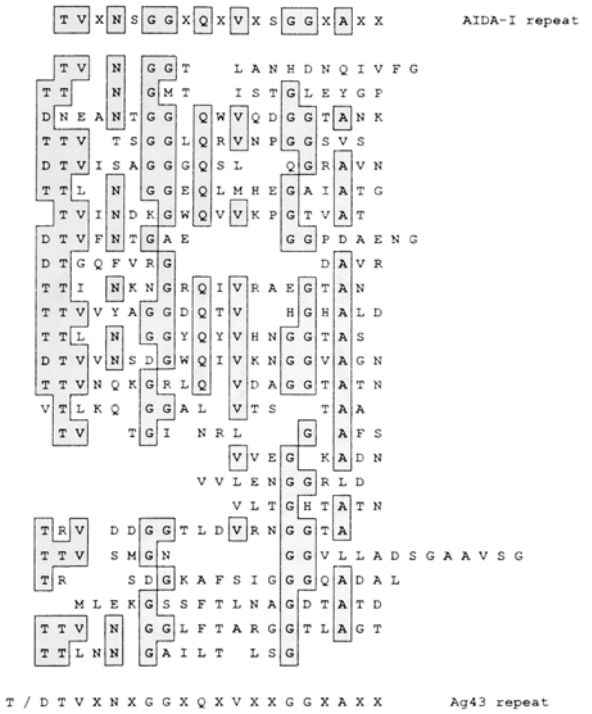


figure II.14. Amino acid sequence of the repeat region of Antigen 43 (From Henderson and Owen, 1999). The consensus sequence of Antigen 43 is shown at the bottom of the figure and the one from AIDA-I is at the top.

carries repetitive sequences similar to the ones seen in AIDA-I. Another way of presenting these repeats is also possible (figure II.15A). In this way, the α segment of Ag43 contains five, approximately 38 bp long, repetitive sequences with relatively high homology, which can be summoned to a consensus sequence (figure II.15B). This consensus sequence seems to be a duplication of the AIDA-I consensus sequence mentioned above. Homology searches against the *E. coli* K-12 (Strain MG1655) database reveals two further open reading frames (*yejO* at 49.2 minutes and *ypjA* at 59.8 minutes on the chromosome), which possess the same consensus sequences (figure II.15C). Both of these are large hypothetical proteins (91.2 kDa and 162 kDa, respectively), which contain putative signal peptides and the signature sequence in the extreme C-terminal which is characteristic for the autotransporters (see figure II.2B). No phenotype has been connected to these large proteins, but these findings suggest, that other outer membrane proteins of the autotransporter family could exist on the *E. coli* surface.

The function of Ag43

Due to the fairly large amount of Ag43 synthesized by *E. coli*, it is reasonable to imagine that it is important for the cell in some way, but nevertheless, a biological role has not been determined yet. A preliminary report has suggested that Ag43 is involved in adherence to Hep-2 cell lines (Owen *et al*., 1996), but this has never been proven. However, the function as an adhesion is tempting, especially when considering the homology to AIDA-I, which promotes diffuse adherence to epithelial cells. As Ag43 expressing cells auto-aggregate and Ag43 thus functions as an auto-adhesin, its role could be to establish multicellular aggregates such as biofilms. Another explanation could be that Ag43 function to remove the cells from the surface of liquid environments, for example to avoid exposure to hazardous UV light. Both of these functions could be connected to the OxyR dependent regulation, as this protein is able to sense oxidative stress within the cells environment. Whatever the biological function, this seems to be exclusively associated to *E. coli* within the members of *Enterobacteriaceae* family (Henderson and Owen, 1999). Interestingly, the aerobic bacterium *Pseudomonas fluorescence* has been shown to exhibit phase variation of both colony morphology and auto-aggregation similar to Ag43-expressing *E. coli*, but the genetic explanation for this is unknown (Rainey and Travisano, 1998).

Potentially, Ag43 could be used as a carrier of foreign epitopes in a manner similar to that seen with other surface components like type 1 fimbriae (Chapter 1) and AIDA-I (Maurer *et al*., 1997). In the latter case, the passenger domain (AIDA-I) has been modified to contain the cholera toxin B subunit and to present this on the cell surface. Alternatively, a completely new passenger domain could be inserted to permit secretion of the new chimeric protein.

Sequence	Amino acid
A Ag43	
NEANTGGQWVQDGGTANKTTVTSGGLQRVNPGGSVSDT	43-80
TTLNGGEQWMHEGAIATGTVINDKGWQVVKPGTVATDT	97-134
TTLNGGYQYVHNGGTASDTVVNSDGWQIVKNGGVAGNT	198-235
VLENGGRLDVLTGHTATNTRVDDGGTLDVRNGGTATTV	290-330
TTVNGGLFTARGGTLAGTTTLNNGAILTLSGKTVNNDT	382-422
B consensus	
***NGG*Q*V**G*TA**T*VN*GG*Q*V**GGVA*DT	Ag43
V*NSGG*Q*V*SGG*A TV*NSGG*Q*V*SGG*A	2 × AIDA-I
C YejO	
TEINGGYQYIEMNGAAEYSVLND-GYQIVQMGGAANQT	76-115
LLENGGSLRVEENDFAYNTTVDSGGLLEVMDGGTVTGV	202-239
D YpjA	
TTINGGRQSIHDGGISTGTTIES-GNQDVYKGGISNGT	143-181
TIINGGTQNINNYGIATGTNINS-GTQNIKSGGKADTT	414-453

figure II. 15. (A) Alignment of the repeats in the α domain of Antigen 43 with position of the amino acid sequences indicated on the right. (B) Consensus sequence (at lease three identical amino acids) derived from (A) compared with a duplicated consensus sequence from AIDA-I. (C) and (D) the similar repeats in the uncharacterized proposed proteins YejO and YpjA from the *E. coli* K-12 (MG1655) chromosome.

This would be advantageous in cases where proteolytic degradation of a protein otherwise impedes its overproduction. As an example, a chimeric protein composed of the FimH protein connected to β fragment of Ag43 could be used as an attempt to overexpress the adhesin of type 1 fimbriae (FimH), which is restricted by periplasmic proteases (Chapter 1).

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Experimental work

Chapter 1: Overexpression of the potential vaccine component, the FimH adhesin, on the surface of *Escherichia coli* by redesign of the *fim* gene cluster.

Chapter 2: Antigen 43 Mediated Auto-Aggregation of *Escherichia coli* is Neutralized by Type 1 Fimbriation.

Chapter 3: The Role of Antigen 43 and Type 1 Fimbriation in *Escherichia coli* Colony Morphology.

Chapter 1

Overexpression of the potential vaccine component, the FimH adhesin, on the surface of *Escherichia coli* by redesign of the *fim* gene cluster

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Summary

Type 1 fimbriae mediate receptor-specific binding of *Escherichia coli* to host surfaces via the FimH adhesin. It is now clear that FimH is critical for the ability of *E. coli* to colonize the urinary tract and is an effective vaccine. This study addresses the issue of FimH expression and mechanisms to enhance the level of surface-located FimH with a view to create a more potent vaccine candidate. This was achieved in a three-step procedure. First, the amount of *fimH* transcript was greatly enhanced by positioning the *fimH* gene immediately behind the *fimA* gene. Next, the amount of periplasmic FimH was enhanced by adding extra copies of the genes encoding the fimbrial transport machinery. Finally, overproduction of surface-located adhesin was achieved by additionally adding extra copies of the minor components. The resultant bacteria produced 25-fold more FimH than did control strains and, because of greatly shortened fimbriae, had an interesting crew-cut appearance. Nevertheless, this strain adhered much better to receptor substrata than did controls. Thus, by rational redesign of the *fim* gene cluster, a potentially improved vaccine strain was created.

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Introduction

One of the decisive factors determining the range of host organisms, tissues and cell types that bacteria are able to colonize is their ability to recognize and bind to specific receptor molecules on host cell surfaces. Fimbriae are one of the major classes of surface structures that are of paramount importance in accomplishing this event. Because they are essential for both commensal and pathogenic strains, the biogenesis, architecture and function of fimbriae has been intensively studied over the past decade and in a number of cases a detailed level of understanding has emerged (see Hultgren *et al*., 1993; Hacker and Morschhäuser, 1994; Klemm and Krogfelt, 1994 for reviews of the field).

Type 1, or mannose-sensitive, fimbriae are found on the majority of *Escherichia coli* strains and are widespread among other members of the *Enterobacteriaceae*. A typical type 1 fimbriated bacterium has 200-500 peritrichously arranged fimbriae on its surface. A single type 1 fimbria is a thin, rod-shaped heteropolymeric structure consisting of four different components that are added to the base of the growing organelle (Lowe and Eisenstein, 1987). Approximately 1000 copies of the major subunit, FimA, are

polymerized into a right-handed helical structure, but small quantities of the minor components, FimF, FimG, and FimH are also present (Klemm and Christiansen, 1987; Krogfelt and Klemm, 1988). It has been shown that the receptor-recognizing element of type 1 fimbriae is the FimH protein (Krogfelt *et al*., 1990).

By virtue of the lectin qualities of the FimH adhesin, type 1 fimbriae mediate adhesion to a variety of mannosylated glycoproteins. Due to the widespread occurrence in both commensal and virulent strains and its highly conserved structure, the FimH adhesin and related adhesiveness was long considered to be of little significance for bacterial virulence. However, due to a number of recent findings this picture has changed dramatically. It has now been clearly demonstrated that the FimH adhesin is critical for the ability of *E. coli* to colonize the urinary tract. *fimH*-null derivatives of highly urovirulent *E. coli* strains were shown to have greatly reduced capacity to initiate infection and to persist in the urinary tract when compared to the parental strains (Connell *et al*., 1996; Langermann *et al*., 1997; Mulvey *et al.*, 1998; Sokurenko *et al*., 1998). Additionally, it has been found that even though FimH is structurally very highly conserved, only minor variations in the *fimH* gene can lead to important phenotypic differences. Indeed, it has been found that there are naturally occurring allelic variants of FimH and those common to uropathogenic isolates appear to be especially effective for the pathogenic colonization of the urinary tract, as compared to those common to fecal isolates (Sokurenko *et al*, 1998). Interestingly, allelic variants of the FimH adhesin found in certain meningitis-causing *E. coli* strains may also be related to virulence (Pouttu *et al*., 1999). The FimH adhesin is thought to mediate attachment and colonization of uropathogenic *E. coli* by recognizing uroplakins, integral membrane glycoproteins of the mammalian urothelium (Wu *et al*., 1996). Expression of type 1 fimbriae on *E. coli* attached in the bladder and kidney has been demonstrated *in situ* (Mulvey *et al*., 1998; Connell *et al*., manuscript in prep.).

The FimH protein is located at the tip in a short tip fibrillum (Jones *et al*., 1995), and, additionally, FimH is interspersed along the fimbrial shaft (Abraham *et al*., 1987; Krogfelt *et al*., 1990). Several lines of evidence suggest that the FimF and FimG proteins act as adapters for integration of the adhesin in the fimbrial organelle (Klemm and Christensen, 1987; Jones *et al*., 1995). Furthermore, the minor components have been suggested to be involved in initiation of fimbrial biogenesis in a dose-dependent manner, determining the length of and number of fimbriae (Klemm and Christiansen, 1987; Russell and Orndorff, 1992; Klemm *et al*., 1994; Jones *et al*., 1995). Like the other structural components of type 1 fimbriae, the FimH adhesin is produced as a precursor with an N-terminal signal sequence, which is subsequently removed during transport across the cytoplasmic membrane. The translocation across the cytoplasmic membrane seems to be dependent on the normal export system (Dodd and Eisenstein, 1984; Pugsley, 1993). However, further export to the cell exterior relies on a fimbriae-specific export and assembly system consisting of the periplasmic FimC chaperone and the outer membrane-located FimD usher protein (Klemm and Christiansen, 1990; Klemm, 1992; Jones *et al*., 1993). Type 1 fimbriae are encoded by a 9.5 kb chromosomal region encompassing the *fim* gene cluster (Klemm *et al*., 1985). Expression is phase-variable with individual cells switching between a fimbriate and a non-fimbriate state by the inversion of a 314 bp DNA segment located immediately upstream of and containing the promoter for the *fimA* gene (Olsen and Klemm, 1994).

Antibodies directed against synthetic peptides mimicking parts of FimH have been shown to

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block attachment of *E. coli* to epithelial cells (Abraham *et al*., 1988) and passive immunization with such antibodies prevented *E. coli* colonization of mouse bladders (Thankavel *et al*., 1997). Recently, it was shown that immunization with purified FimH could prevent urinary tract infection by *E. coli* in the murine model (Langermann *et al*., 1997). The protective effect of the anti-FimH antibodies provided additional evidence for the significance of the FimH adhesin as a virulence factor and calls for further exploration of various approaches for the design of FimH vaccine candidates. In the present study we have investigated the possibility of redesigning the *fim* gene cluster in order to construct *E. coli* strain which overexpress the FimH adhesin on the surface. Such strains could serve a number of useful purposes. A strain overexpressing FimH could facilitate FimH purification and could be valuable for creating attenuated live vaccines as alternatives to FimH component vaccines.

Results

Transcription from the fim gene cluster

It was previously found that the transcription of type 1 fimbriae genes is driven by a promoter located within the phase switch with a transcript start 132 bp upstream of the *fimA* gene (Olsen and Klemm, 1994). To gain further insight into the level of *fimH* expression compared to that of the other genes of the *fim* cluster, transcriptional fusions spanning the entire *fim* gene cluster were constructed, fusing *lacZ* with *fimA*, *fimI*, *fimD* or *fimH* (Fig. 1A).

Significant β-galactosidase activity was observed with the *fimA-lacZ* fusion, but at each successive downstream reporter site expression levels diminished dramatically. Notably, β-galactosidase activities originating

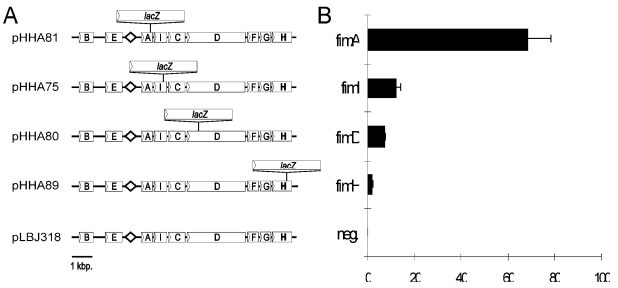


Fig. 1. β-galactosidase activities of transcriptional *lacZ* fusions in four *fim* genes.
A. Schematic representation of insertion points of the *lacZ* gene.
B. β-Galactosidase values (in Miller units) of cells harboring one of the five plasmids shown in A. Error bars indicate the standard deviations based on 6 measurements.

from the *fimA-lacZ* and the *fimH-lacZ* fusions differed by approximately 50-fold (Fig. 1B). Therefore, it appears that the ratio of the different *fim* gene products is regulated to a large degree at the transcriptional level.

Juxtaposition of the fimH and fimA genes and modification of the ribosome-binding site.

In the natural *fim* gene cluster, the *fimA* gene is flanked by two dyad symmetry segments located approximately 90 bp upstream and 50 bp downstream of *fimA*. Since such motifs can enhance the stability of the intervening transcript (McLaren *et al.*, 1991; Emory *et al.*, 1991) these dyad symmetry segments may contribute to the extraordinarily high level of the FimA protein relative to the other fimbrial subunits. Thus, we tested the effects of relocating the *fimH* gene in close proximity to the *fimA* gene on the expression of the FimH subunit. To rule out potential expression from the original *fimH* gene, plasmid pPKL115, which harbors a translational stop-linker in the wild-type *fimH* gene, was used as the basis for the new constructions. During the creation of these redesigned *fim* clusters, the *fimH* ribosomal binding site was also modified by site-directed mutagenesis (see Experimental Procedures section). This was done because, while the Shine-Dalgarno sequence of *fimA* (5'-

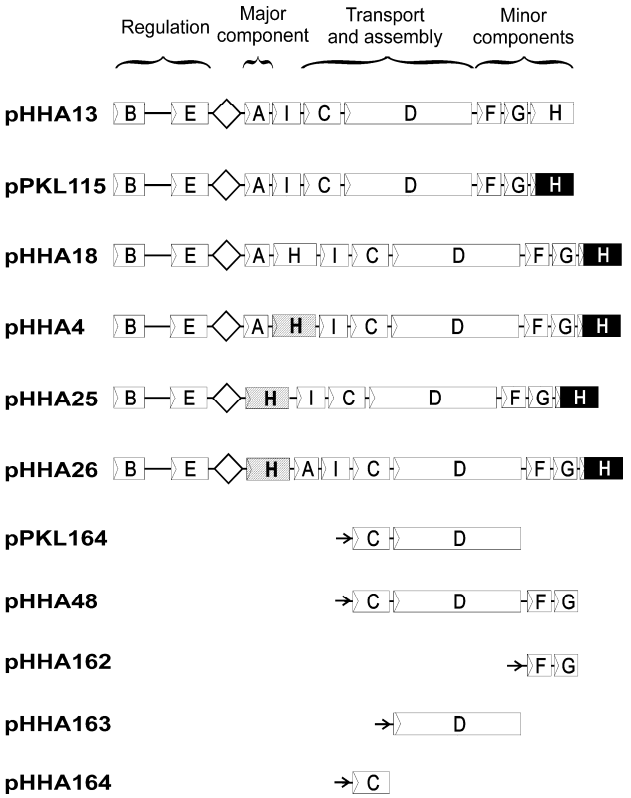


Fig. 2. Overview the wild type and engineered versions of the *fim* gene cluster. White chevrons indicate the transcriptional orientation of the individual genes. Black versions of the *fimH* gene indicate the presence of a translational stop linker. Hatched versions of the *fimH* gene indicate the presence of a modified ribosomal binding site. A diamond indicates the promoter residing in the *fim* switch.

AAGGAA-3') is very close to the theoretical optimum for interactions with the 3'-end of ribosomal 16S rRNA (Gold and Stormo, 1996), the ribosome binding site of *fimH* (5'-GGGATG-3') is not.

A transcript with an apparent length of approximately 850 bases was identified in Northern blots probing RNA isolated from a host containing the wild-type *fim* gene cluster (MS7(pHHA13); Fig. 2) with a *fimA*-specific probe (Fig. 3A, lane 2). The size of this transcript is in reasonable agreement with the size predicted for the *fimA*-transcript containing two stem-loop structures (i.e. 750 bp; see above). Under the same conditions a *fimH*-specific probe was unable to detect any transcript from MS7(pHHA13) RNA, indicating a very low level of such an RNA species. In two plasmid constructs (i.e. pHHA26 and pHHA4, respectively), the *fimH* gene with an improved ribosome- binding site was positioned immediately upstream or downstream of the *fimA* gene (Fig. 2). RNA from *E. coli* bearing pHHA26 did not hybridize with either the *fimA*- specific or the *fimH*-specific probe (Fig. 3A & 3B, lane 4). It may be noteworthy in this regard that the *fimH* gene contains multiple potential RNase E endonuclease cleavage sites, whereas the *fimA* gene has none. Although the rules governing RNase E-mediated degradation are somewhat unpredictable (Cohen *et al*, 1997), the proximity of the *fimH* gene to the transcript start site in this plasmid may explain the apparent transcript instability. In any case, plasmid pHHA26 was not utilized in any of the additional experiments. Northern blotting of RNA isolated from a host harboring plasmid pHHA4, on the other hand, revealed the presence of a transcript of approximately 1.8 kb that reacted with both the *fimA* and the *fimH* probes (Fig. 3A and 3B, lane 3). This result is in agreement with the predicted size of 1700 bases for a transcript harboring message from both *fimA* and *fimH*. Importantly, this result indicates that the level of *fimH*-containing mRNA is dramatically increased in MS7(pHHA4) compared to the wild-type

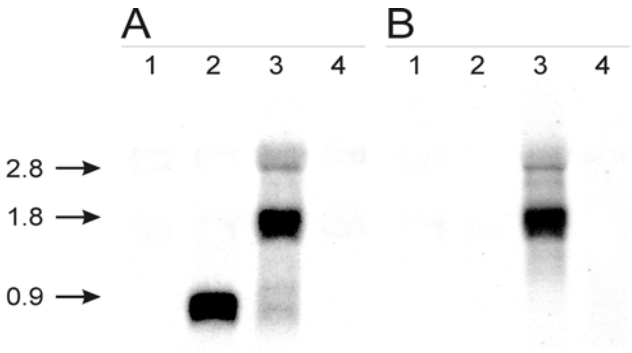


Fig. 3. Northern hybridization analysis, employing a *fimA*-specific probe (panel A) and a *fimH*-specific probe (panel B), of transcripts originating from a *Δfim* MS7 host (lane 1) or the same host containing plasmids pHHA13 (lane 2), pHHA4 (lane 3), and pHHA26 (lane 4). Transcript sizes indicated in Kb pairs.

MS7(pHHA13).

Despite elevated *fimH* mRNA levels in

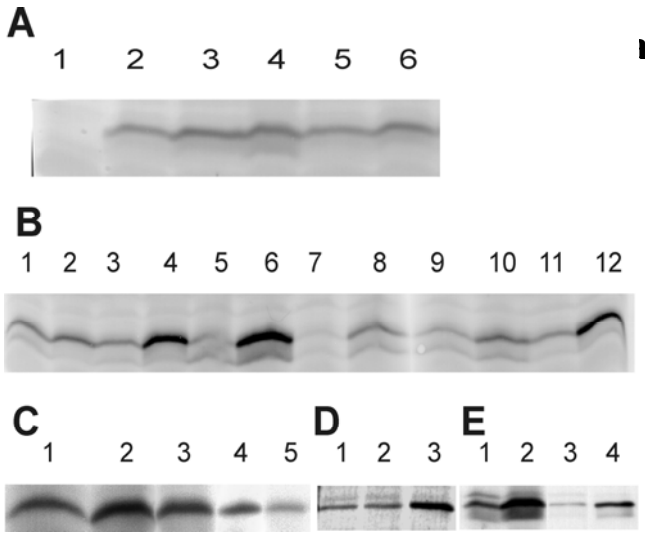


Fig. 2. Receptor blot analysis of Δ*fim* MS7 (4A-D) and Nissle 1917 (4E) hosts. Total cell lysates were subjected to SDS-PAGE and protein blotting and assayed for the presents of FimH by adding mannose BSA, anti BSA and horseradish peroxidase (HRP) labeled Pig anti-rabbit IgG.

A. MS7 cells carrying pPKL115 (*fimH* $^{-}$; lane 1), pHHA13 (wild type; lane 2), pHHA18 (lane 3), pHHA4 (lane 4), pHHA25 (lane 5) pHHA26 (lane 6).
B. MS7 cells carrying pHHA13 (lane 1), pHHA4 (lane 2), pHHA13/pPKL164 (lane 3), pHHA4/pPKL164 (lane 4), pHHA13/pHHA48 (lane 5), pHHA4/pHHA48 (lane 6), pHHA13/pHHA162 (lane 7), pHHA4/pHHA162 (lane 8), pHHA13/pHHA163 (lane 9), pHHA4/pHHA163 (lane 10), pHHA13/pHHA164 (lane 11) and pHHA4/pHHA164 (lane 12).
C. Dilutions of cell lysates from MS7(pHHA4/pHHA48) hosts compared to undiluted cell lysates from hosts carrying pHHA13 (wild type gene cluster). pHHA13: undiluted (lane 1), pHHA4: dilution 1:10 (lane 2), dilution 1:20 (lane 3), dilution 1:30 (lane 4), and dilution 1:40 (lane 5).
D. Aliquots of precipitated culture media from MS7 hosts pHHA13 (lane 1), pHHA13/pHHA48 (lane 2) and pHHA4/pHHA48 (lane 3).
E. *E. coli* Nissle 1917(pHHA13) cells (lane 1) and precipitated culture media from these (lane 3) compared with Nissle 1917 (pHHA4/pHHA48) cells (lane 2) and precipitated culture media from these.

MS7(pHHA4), receptor blotting of whole cell lysates indicated that there was no detectable increase in the amount of the FimH adhesin over wild-type FimH levels in MS7(pHHA13) (Fig. 4A, lanes 2 and 4).

Influence of the export machinery dose

To investigate whether increasing the relative levels of the components of the fimbrial transport machinery would lead to increased levels of FimH, we introduced into selected hosts a second plasmid, pPKL164, encoding the *fimC* chaperone and the *fimD* usher (Fig. 2). Transcomplementation of plasmid pHHA13 containing the wild-type *fim* gene cluster with pPKL164 did not result in any detectable increase in the FimH level as assayed by receptor-blotting of whole cell lysates (Fig. 4B, lane 3). However, introducing pPKL164 into MS7(pHHA4) resulted in a significant increase in the FimH level (Fig. 4B, lane 4). To examine the individual contributions made by FimC or FimD, two plasmids encoding the corresponding genes (i.e. pHHA163 and pHHA164, respectively) were created (Fig. 2). Introduction of these plasmids into relevant hosts revealed that increasing the amount of FimD was reflected in increased FimH levels in whole cell extracts (Fig. 4C, lane 9 and 10), but increasing the amount of the FimC chaperone resulted in significantly more FimH (Fig. 4B, lanes 9-12).

We then examined whether the enhanced level of FimH adhesin in MS7(pHHA4; pPKL164)

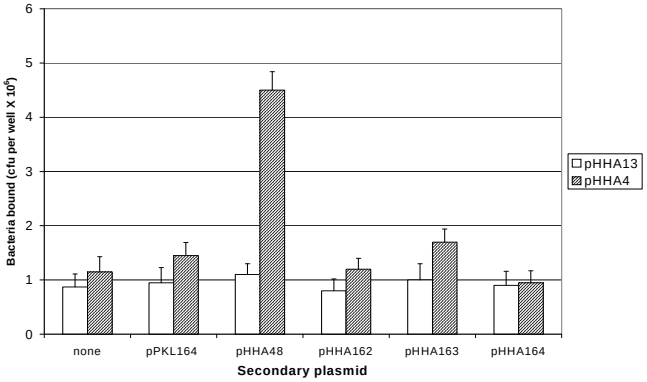


Fig. 5. Adhesion assay to yeast mannan. Bars represent cells carrying either a wild type gene cluster (white) or a modified gene cluster (hatched) in concert with complementing plasmids. pPKL164: extra *fimC* and *fimD*, pHHA48: extra *fimC, fimD*, *fimF,and fimG*, pHHA162: extra *fimF* and *fimG*, pHHA163: extra *fimD*, pHHA164: extra *fimC*.

would be reflected in a corresponding increase in the amount of FimH on the bacterial surface. This was tested in two ways, first by determining the adhesive capacity of the bacterial host toward yeast mannan, a classical receptor target for FimH (Fig. 5), and second by immunofluorescence employing anti-FimH antibodies (Fig. 6). We observed little or no

difference between MS7(pHHA4; pPKL164) and the control
strains in either of these assays for surface expression. Therefore, the increased amounts of components of the fimbrial transport machinery results in protection of FimH from proteolytic degradation in the periplasm, but does not enhance presentation of the adhesin on the bacterial surface.

Influence of additional FimF and FimG

As previously mentioned, several lines of evidence indicate that the two other minor constituents of type 1 fimbriae, FimF and FimG, contribute in important ways to the integration of the adhesin into the fimbrial organelle. Therefore a plasmid encoding the *fimF* and *fimG* genes in addition to the genes encoding the transport machinery (pHHA48; Fig. 2) was introduced into hosts harboring plasmid pHHA4. Receptor blots of whole cell extracts of these strains indicated that there was an approximately 25-fold increase in FimH activity compared to strains not containing plasmid pHHA48 (Fig. 4B, lanes 2 and 6 and Fig. 4C). Furthermore, MS7(pHHA4; pHHA48) had a much greater ability to adhere to yeast mannan than did the relevant control, suggesting increased levels of the functional FimH adhesin on the bacterial surface (Fig. 5). Immunofluorescence studies also revealed an approximately 10-fold difference in signal strength between MS7(pHHA4; pHHA48) and control cells (Fig. 6). These results indicated that increased amounts of both the transport machinery and minor fimbrial components were necessary to significantly enhance the surface presentation of the FimH adhesin. Transcomplementation of MS7(pHHA4) with a plasmid bearing *fimF* and *fimG* and without

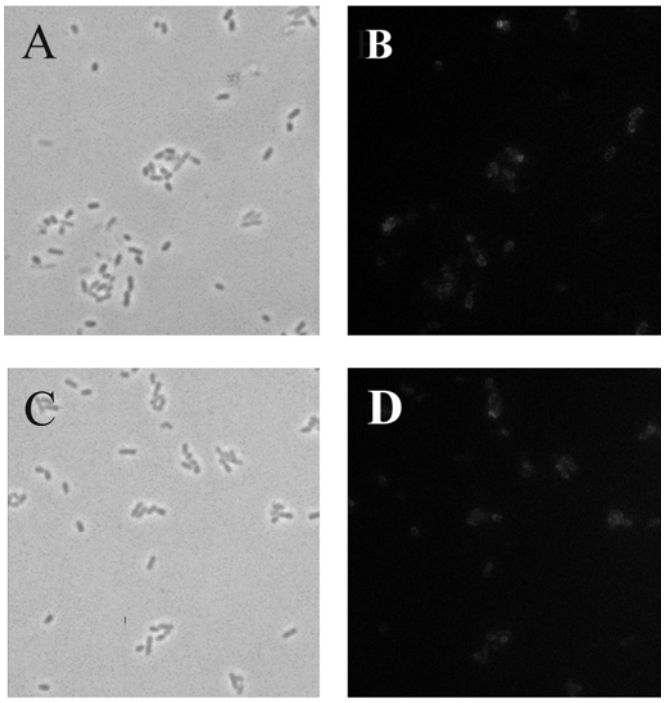


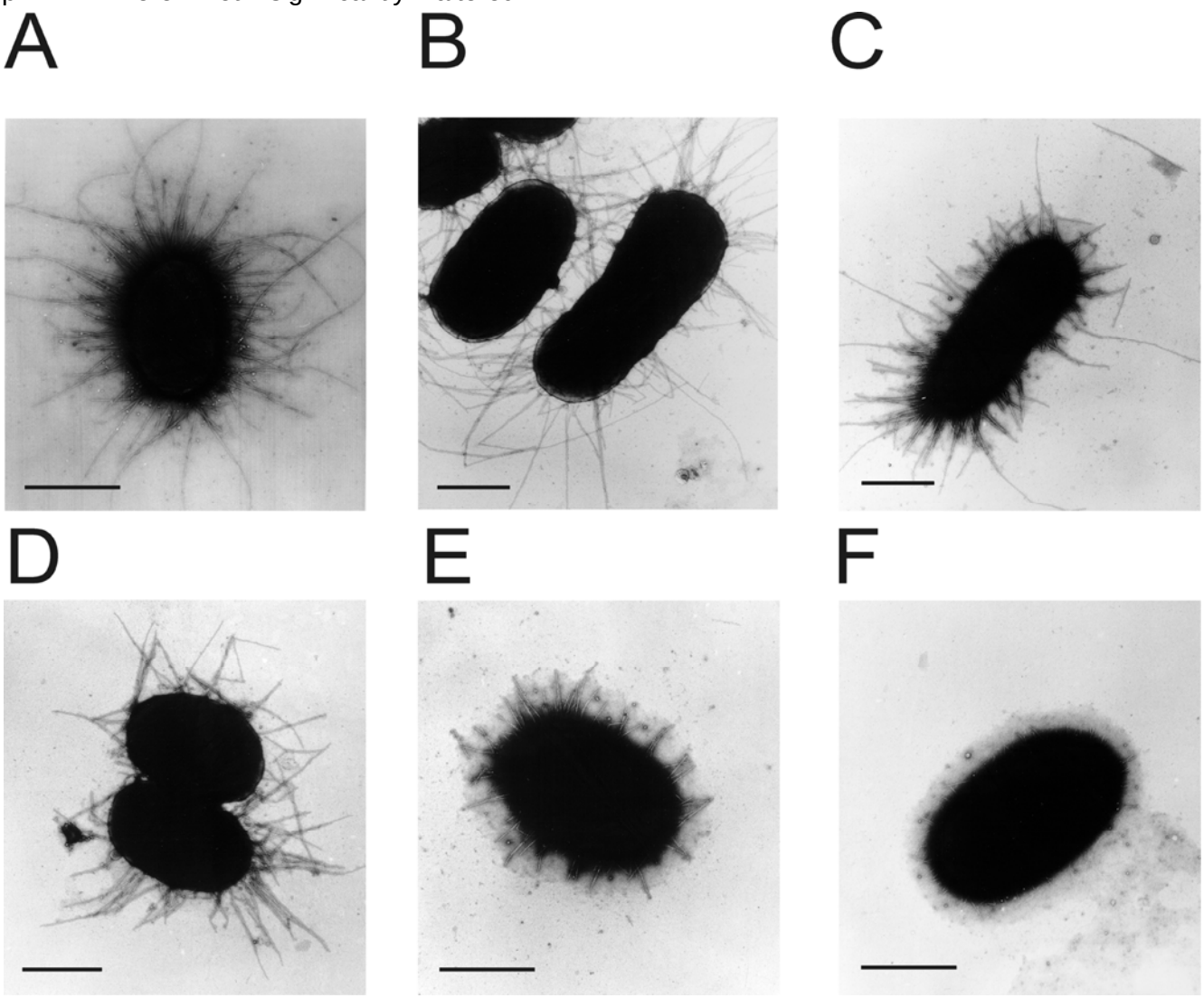
Fig. 6. Phase contrast microscopy (PCM) and Fluorescence microcopy (FM) of Δ*fim* MS7 hosts. In all cases a monoclonal antibody that is known to recognize a conformational epitope in FimH was used as primary antibody and a FITC labeled rabbit anti mouse IgG and a FITC labeled pig anti rabbit IgG as secondary and tertiary antibodies.
A and B. PCM (A) and FM (B) of *E. coli* cells carrying a plasmid with the wild type gene cluster (pHHA13).
C and D. PCM (C) and FM (D) of *E. coli* cells carying a plasmid with a modified gene cluster (pHHA4) in concert with a plasmid expressing FimC and FimD (pPKL164).
E and F. PCM (E) and FM (F) of *E. coli* cells carying a plasmid with a modified gene cluster (pHHA4) in concert with a plasmid encoding the *fimC*, *fimD*, *fimG* and *fimF* genes (pHHA48).

fimC or *fimD* (pHHA162; Fig. 2) did not result in either elevated amounts (Fig. 4) or surface expression (Fig. 5) of FimH. The elevated level of FimH on the surface of MS7(pHHA4;pHHA48) was also reflected in increased amounts of the adhesin shed in the growth medium. This was tested by receptor-blotting of acetone-precipitates of growth medium of this strain and relevant control strains (Fig. 4D).

Fig. 7. Electron micrographs of Δ*fim* MS7 hosts containing the following plasmids: A – C. pHHA13 (A), pHHA13 plus pPKL164 (B) and pHHA13 plus pHHA48 (C). D - F. pHHA4 (D), pHHA4 plus pPKL164 (E) pHHA4 plus pHHA48 (F). Bar indicates 0.5 μm.

Fimbrial Morphology

Bacterial hosts bearing a plasmid encoding the wild-type *fim* gene cluster express several hundred fimbriae with lengths of approximately 0.5 -1 μm (Fig. 7A). *E. coli* bearing plasmid pHHA4 were not significantly altered in appearance (Fig. 7D). However, increasing the transport machinery dose by introduction of plasmid pPKL164 (*fimC*$^+$, *fimD*$^+$) resulted in cells with notably shorter fimbriae (Fig. 7E). A very much shorter, crew-cut fimbrial phenotype was observed when plasmid pHHA4 was complemented with pHHA48 (*fimC*$^+$ - *fimG*$^+$) (Fig. 7F). Combining the wild-type control plasmid with plasmid pPKL164 or pHHA48, however, did not result in a similarly dramatic change in fimbrial morphology, although a definite tendency toward somewhat shorter fimbriae was observed with MS7(pHHA13; pHHA48) (Fig. 7C).



Discussion

Over the last few years our perception of the role

of type 1 fimbriae and the fimbrial adhesin, FimH, in bacterial pathogenesis has changed considerably. Recent studies have now firmly established the FimH adhesin as an important virulence factor of uropathogenic and perhaps also of meningitis-causing *E. coli* strains (Connell *et al*., 1996; Wu *et al*., 1996; Mulvey *et al*., 1998; Sokurenko *et al*., 1998; Pouttu *et al*., 1999). FimH is a highly versatile adhesin in which single amino acid substitutions can lead to dramatic changes in receptor specificity. This functional flexibility might well be a prominent aspect of the capacity of FimH as a virulence factor.

The potential of FimH as a vaccine component has been tested and proven to be highly efficient in animal models (Langermann *et al*., 1997). These authors demonstrated that purified complexes of FimH and the FimC chaperone and purified FimH truncates can be used for systemic immunization with significant success. It is known, however, that using recombinant bacterial vectors which express vaccine antigen(s) at high level can have certain advantages over purified component vaccines (Walker, 1994; Lindberg and Pillai, 1996). In addition to the low cost and easy mucosal route of administration of live, attenuated bacteria, the ability of these organisms to colonize mucosal surfaces and mimic, at least in part, a natural infection, may elicit a more appropriate mucosal immune response. Because the FimH adhesin has the ability to bind to mucosal surfaces, it may belong in the same category as the so-called adhesive antigens that have found to be highly active in mucosal vaccination (Walker, 1994). It has also been shown that heterologous epitopes can be inserted into FimH without loss of functional activity (Pallesen *et al*., 1995) and thus may allow delivery of these heterologous vaccine antigen epitopes as well. It is with this background that we began to investigate the feasibility of engineering *E. coli* which overexpress the FimH adhesin on the surface.

A highly choreographed series of protein-protein interactions ultimately leads to assembly of a fimbrial organelle on the bacterial surface. The composition of the organelle must depend on such factors as the affinity of the individual structural proteins with the components of the fimbrial transport machinery and the concentrations of the subunit proteins. In turn the amount of the individual gene products is governed by factors such as the position of the genes within the cluster, transcript stability, and the translation efficacy. In the wild type scenario, the final outcome is orchestrated to result in a surface organelle where the bulk of the structure is composed of FimA with only small quantities of FimF, FimG and FimH. This composition is probably a structural compromise to fit the bacteria's requirements in various environmental niches. However, it is obviously not ideal if one wishes to express large amounts of FimH. Abraham *et al*. (1988) previously reported on a mutant that produced high levels of FimH adhesin and this was thought to be due to the deletion of a 300 bp fragment downstream of the *fimH* gene. It was suggested that the sector downstream of FimH encoded an element, which repressed expression of *fimH* and/or other *fim* genes. Unfortunately we have been unable to evaluate the fimbriosome-producing strain and it has apparently been lost (S. N. Abraham, personal communication). Furthermore, no reference to this strain has occurred in the literature since it was published. The so-called "fimbriosomes" (Abraham *et al*., 1988) do not bear an ultrastructural similarity to the FimH-containing fimbriae and fimbrial fragments reported here. In earlier efforts to overexpress FimH, we were never able to reproduce the "fimbriosome" effect by deleting portions of the region downstream of *fimH*. In fact, in the strains utilized in our studies, the gene located immediately downstream of *fimH*, *gntP*, encodes

a gluconate permease, a protein having a function that would appear to be quite distant from regulation of fimbrial expression (Klemm *et al*, 1996). In this report, we redesigned the *fim* gene cluster to explore ways to overexpress FimH on the bacterial surface and were able to accomplish the following steps in recombinant bacteria: (i) the level of the *fimH* transcript was enhanced, (ii) the stability of FimH protein was enhanced, and (iii) the surface expression of functional FimH was enhanced.

The transcriptional activity of *fim* genes located downstream of *fimA* had not previously been monitored, but it is clear from the results presented herein that at least part of the reason for the very limited expression of FimF, FimG and FimH is low transcript level. There was a 50-fold difference in the β-galactosidase activities originating from the reporter cassette positioned in *fimA* and that in *fimH*. This result was corroborated by Northern blot analysis in which a strong signal was detected using a *fimA* probe and no signal was detected using a *fimH* probe. When the *fimH* gene was positioned immediately downstream of *fimA*, as in plasmid pHHA4, a double-cistronic transcript could be readily detected with both probes. We believe that site-directed mutagenesis of the ribosome-binding site of *fimH* to generate the same ribosome-binding site structure as that of *fimA* also added to increased *fimH* message, but this cannot be determined with certainty from the constructs reported here.

Although it is clear that *fimH* message dramatically increased, these two endeavors nevertheless did not result in significantly more detectable FimH protein in whole-cell extracts compared to the wild type system. Therefore, we introduced additional copies of the genes for the fimbrial transport machinery, FimC and FimD, and genes for the minor fimbrial components FimF and FimG. Additional copies of *fimC*, but not *fimD*, in cells harboring plasmid pHHA4 resulted in significantly higher levels of intact FimH in whole-cell extracts, but extra *fimC* or *fimC* and *fimD* together did not lead to increased FimH on the bacterial surface.

The goal of overexpression of surface-bound FimH was achieved only when extra copies of *fimC*, *fimD*, *fimF* and *fimG* (pHHA48) were supplied to cells bearing plasmid pHHA4. Evidence supporting this conclusion includes receptor blotting data indicating that there was approximately 25 times more FimH than with the wild-type *fim* cluster, adhesion assay data indicating an approximately 5-fold increased adhesion to a mono-mannose substratum than any of the relevant controls and immunofluorescence data showing a much better binding of anti-FimH antibodies than with controls. Interestingly, electron microscopy of MS7(pHHA4; pHHA48) disclosed a dramatic shortening of cell-bound fimbriae and the presence of numerous fimbrial “fragments” in the medium. Indeed, the growth medium of such cells was found to contain significant amounts of FimH (Fig. 4D). This phenotype supports a model of fimbrial biogenesis in which a complex consisting of the adhesin in addition to FimF and/or FimG initiates the organelle assembly on the bacterial surface as suggested in previous reports (Klemm and Christiansen, 1987; Klemm *et al*., 1994; Jones *et al*., 1995). However, this phenotype is not inconsistent with the concept that FimF, FimG and FimH complexes are added at intervals during fimbrial biogenesis and that the points where these complexes occur are regions of fimbrial fragility, as suggested previously (Klemm and accompanied by an increased sensitivity to mannose inhibition (data not shown) that is typical for monomannose-specific variants of FimH (Sokurenko *et al*. 1997).

In the present study we have achieved overproduction and surface presentation of the FimH adhesin by rational redesign of the *fim* gene cluster, especially in the case of MS7(pHHA4; pHHA48). Increased FimH expression accompanied by the dramatically

shortened fimbriae expressed by this strain suggest that the FimH:FimA ratio is greatly increased compared to wild-type. This characteristic may be beneficial for the construction of a live vaccine strains, since the immune response to FimA is not considered to be as protective as an immune response against FimH (Langermann *et al*., 1997). Since *E. coli* K-12 strains might not be ideal as live vaccine candidates we also tested the FimH overexpression system in a more relevant host strain, viz. *E. coli* Nissle 1917. This strain is an excellent colonizer of the human gut and has been extensively used as a probiotic (Lodinova-Zadnikova, 1992; Schulze and Sonnenborn, 1995). In line with the results obtained in K-12 strains it was found that FimH overproduction could also be achieved in the Nissle 1917 strain (Fig. 4E).

Although we have addressed some of the more likely bottlenecks inherent in FimH expression from the wild-type gene cluster (e.g. transcript stability and dosage of auxiliary proteins), other factors could be addressed to achieve further optimization. One such factor is codon usage. The codon usage in the *fimH* gene is approximately 65% of the theoretical optimum according to Hénaut and Danchin (1996), whereas that for *fimA* is approximately 80%. It is conceivable that a transcript originating from a synthetic *fimH* gene with optimized codon usage would be translated more efficiently. Also, the middle sector of the FimH protein is prone to proteolytic processing in the periplasm, resulting in two truncates of approximately equal size (Jones *et al*., 1993; Jones *et al*., 1995; Knudsen and Klemm, 1998). Alteration of this site might also lead to additional increases in the level of FimH expression.

Although there are other issues that will need to be addressed in the future, the present study demonstrates that some of the major requirements for creation of a live FimH vaccine strain can be met by rational re-design of the type 1 fimbrial gene cluster. FimH can be overexpressed on the bacterial surface, increasing antigen delivery by the bacterial vector; the surface FimH retains functional activity and could provide bacteria with increased tropism for mucosal surfaces. We have also reported previously on the construction and expression of chimeric versions of FimH subunits that contain foreign key epitopes from cholera toxin and hepatitis B surface antigen (Pallesen *et al*., 1995). Following the same rationale used here, such constructs could be overexpressed in a relevant host, resulting in interesting polyvalent vaccines. Furthermore, FimH was recently engineered to display peptides that specifically bind to heavy metals, such as cadmium and lead (Schembri and Klemm, 1998; Schembri *et al*., 1999). The overexpres-sion system described here could be used to upgrade expression of such metal-binding chimeras on the cells, thus enhancing the capacity to sequester toxic metals, for instance in the bioremediation of metal-polluted water.

Experimental procedures

Bacterial strains and growth conditions

Chapter 1

A list of *E. coli* strains used in this study is provided in Table 1. Cells were grown on solid medium or in liquid broth supplemented with the appropriate antibiotics unless otherwise stated.

Plasmids used in this study are listed in Table 1. Plasmid pHHA13 was made by ligating a9995 bp *Hin*dIII-*Eag*I fragment from pPKL4 (Klemm *et al*., 1985) into the same sites in pACYC184. Plasmids pHHA81, pHHA75 and pHHA89 were made by inserting a 3946 bp *Pst*I

Table 1. *E. coli* Strains and plasmids used in this study

Strain or plasmid	Genotype/Description	Reference
Strains		
VL751	*E. coli* K-12 (*ara* Δ(*lac-pro*) *rpsL thi* Δ*fim*)	Freitag *et al*., 1985
MS7	*E. coli* K-12 strain PC31 (*gal, tonA, phx, argF, rel,* Δ*fimB-H, recA, Kan*^{R})	Stentebjerg-Olesen *et al*., 1997
Nissle 1917	*E. coli* strain Nissle 1917 of serotype O6:K5:H1 (*fim*$^+$)	Blum *et al*., 1995.
Plasmids		
pHHA4 (A)	959 bp PCR fragment containing a *fimH* gene with a modified ribosome binding site amplified from pPKL4 using primers 1 & 2, digested with *Aat*II and inserted into the *Aat*II site of pPKL115	This study
pHHA13 (A)	9995 bp *Hin*dIII/*Eag*I fragment containing the *fim* gene cluster from pPKL4 inserted into the same sites of pACYC184	This study
pHHA18 (A)	961 bp PCR fragment containing a *fimH* gene with it's normal ribosome binding site amplified from pPKL4 using primers 3 & 2 was digested with *Aat*II and inserted into the *Aat*II site of pPKL115	This study
pHHA22 (B)	642 bp *Sac*I fragment from pLPA41 containing the *fimA* gene was inserted into the *Sac*I site of pHHA23	This study
pHHA23 (B)	Artificial polylinker containing an *Aat*II and an *Eag*I site flanked by *Sac*I compatible overhangs inserted into pLBJ312 generating one *Sac*I site.	This study
pHHA24 (A)	5610 bp *Hin*dIII/*Bam*HI fragment containing *fimB-D'* from pHHA22 ligated to the same sites of pPKL115	This study
pHHA25 (A)	Deletion of a 642 bp *Sac*I fragment containing the *fimA* gene from pHHA26	
pHHA26 (A)	959 bp AatII fragment containing a *fimH* gene with a modified ribosome binding site from pHHA4 inserted into the *Aat*II site of pHHA24	This study
pHHA48 (B)	4436 bp *Bam*HI/*Eag*I fragment containing *fimD'FG* from pHHA18 ligated into the same sites of pPKL164	This study
pHHA75 (B)	Blunt 3946 bp *Pst*I fragment from pCB267 containing a promotorless *lacZ* gene inserted into the *Stu*I site in *fimI* of pLBJ318.	This study
pHHA80 (B)	3936 bp *Bgl*II/*Bam*HI fragment from pCB267 containing a promotorless *lacZ* gene inserted into the *Bam*HI site in *fimD* of pLBJ318.	This study
pHHA81 (B)	3946 bp *Pst*I fragment from pCB267 containing a promotorless *lacZ* gene inserted into the *Pst*I site in *fimA* of pLBJ318.	This study
pHHA89 (B)	3946 bp.*Pst*I fragment from pCB267 containing a promotorless *lacZ* gene inserted into the *Pst*I site in *fimH* of pLBJ318.	This study
pHHA162 (B)	pHHA48 digested with *Eco*RV and religated. This deletes *fimC* and *fimD* and brings *fimF* and *fimG* next to the *tet* promoter.	This study
pHHA163 (B)	pPKL164 digested with *Sna*BI and *Sma*I and religated. This deletes 282 bp from the *fimC* gene.	This study
pHHA164 (B)	pPKL164 was digested with *Mlu*I/*Bam*HI blunted and religated. This deletes 263 bp from *fimD* gene.	This study
pLBJ312 (B)	Wild type *fim* gene cluster where *fimA* has been deleted and the *Aat*II site behind *fimA* is substituted with a *Sac*I site.	
pLBJ318 (B)	642 bp fragment of pLPA41containing *fimA* flanked by *Sac*I sites was inserted into the *Sac*I site of pLBJ312.	Stentebjerg-Olesen *et al*. (1997)
pLPA41 (P)	PCR fragment containing the *fimA* gene flanked by *Sac*I sites inserted into the *Sac*I site of pUC18.	Stentebjerg-Olesen *et al*. (1997)
pPKL4 (B)	*fim* gene cluster of *E. coli* strain PC31 on pBR322.	Klemm *et al.* (1985)
pPKL115 (A)	*fim* gene cluster of *E. coli* strain PC31 with a translational stoplinker in the *Kpn*I site of *fimH*.	Pallesen *et al.* (1995)
pPKL164 (B)	*fimC* and *fimD* from pPKL4 inserted into the *tet* gene of pBR322.	Klemm *et al.* (1995)

A, B and P in brackets indicate pACYC184, pBR322 and pUC19 vectors.

Plasmids

fragment from pCB267 (Schneider and Beck, 1986) containing the promoterless *lacZ* gene including its ribosome binding site into the *Pst*I site in *fimA*, the *Stu*I site in *fimI* and the *Pst*I site

of *fimH*, respectively, of plasmid pLBJ318. Plasmid pHHA80 was made in the same way as the three plasmids above, but the *lacZ* fragment was a 3936 bp *Bam*HI-*Bgl*II and this was inserted into the *Bam*HI site (in *fimD*) of pLBJ318 (Fig. 2). Plasmid pHHA4 was made by inserting a 959 bp PCR fragment containing the *fimH* gene modified to have the ribosomal binding site of *fimA* and flanked in both ends by *Aat*II sites into the unique *Aat*II site of pPKL115. The PCR fragment was made employing primers 1 and 2 and plasmid pPKL4 as template. Plasmid pHHA18 is equivalent to pHHA4, but in this case the *fimH* gene has the normal ribosomal binding site. Plasmid pHHA23 was made by inserting a poly linker (containing an *Aat*II and a *Eag*I site flanked by *Sac*I overhangs) into the unique *Sac*I site of pLBJ312. Plasmid pHHA22 was constructed by inserting the *Sac*I fragment, containing *fimA*, from plasmid pLPA41 into the *Sac*I site in the poly linker of pHHA23. pHHA26 was made by joining the 5.6 kb *Eco*RI-*Hin*dIII fragment of plasmid pHHA22 with the 7.8 kb *Eco*RI-*Hin*dIII fragment of pPKL115. Plasmid pHHA25 was made by deleting the *fimA*-containing *Sac*I fragment from pHHA26.

DNA techniques

Isolation of plasmid DNA was carried out using the QIAprep Spin Miniprep Kit (Qiagen). Restriction endonucleases were used according to the manufacturer's specifications (Biolabs).

PCR methodology

Polymerase chain reactions were made as previously described (Stentebjerg-Olesen *et al*., 1997). The following primers were used:
1:5'GCGGACGTCAAAGGAAAGCAGCATGAAACGAGTTATTACCCTG **2**:5'CGCGACGTCGCATTAGCAATGTCCTGTGATTTC **3**:5'GCGGACGTCGAGGGATGATTGTAATGAAACGAG.

Nucleotide sequencing

The nucleotide sequences of PCR products and flanking regions in the genetic constructs were determined by using the ABI PRISM^{TM} BigDye Terminator cycle sequencing ready reaction kit (PE Applied biosystems). Samples were electrophoresed on a Perkin-Elmer ABI PRISM 310 Genetic Analyzer (PE Applied biosystems) as described in the manufacturer's specifications.

Beta-galactosidase assay

E. coli VL751 cells containing either plasmid pHHA75, pHHA80, pHHA81 or pHHA89, respectively, were grown in AB minimal media (Clark and Maaløe, 1967) supplemented with 1 µg/ml thiamin, 0.2 % glucose, proline (80 µg/ml), valine, leucine and isoleucine (50 µg/ml) and ampicillin (100 µg/ml). The four strains were grown exponentially for at least 10 generations and the assay was performed as described by Miller (1992).

RNA isolation and Northern (RNA) blot hybridization

Total RNA was isolated from *E. coli* strain MS7, MS7(pHHA13), MS7(pHHA18), and MS7(pHHA26). Isolation of RNA, electrophoresis, blotting to the membrane (QiaGen, Hilden, Germany), hybridization and development were carried out as described by Nygaard *et al*. (1996). *fimA* and *fimH* DNA probes were labeled with [γ-^{32}P]-dATP using a random-primer labeling kit (Boehringer).

Precipitation of proteins from growth media

Total protein from 2 ml of culture media was acetone precipitated by adding 6 ml of cold acetone and spinning the samples for 30 minutes (4 °C) at 20,000 * *g* on a microcentrifuge and resuspending the samples in 200 µl 0.9 % NaCl.

Receptor blotting

Receptor blots of *E. coli* cultures and precipitated growth media containing FimH to D-mannosylated bovine serum albumin (Sigma) were made essentially as previously described (Krogfelt *et al*., 1990).

Adhesion assay

Adhesion assays were performed in 96 well micro titer plates coated with yeast mannan as described previously (Sokurenko *et al*., 1997).

Immunofluorescence microscopy

Surface presentation of FimH was assessed by immunofluorescence microscopy employing a monoclonal antibody that recognizes a conformational epitope in FimH (Chanteloup *et al*., 1991). Cell fixation, immunolabeling and microscopy was basically carried out as previously described (Pallesen *et al*., 1995) with the exception that a FITC-labeled tertiary antibody was used in addition to the secondary antibody.

Electron microscopy

Bacteria from overnight cultures were resuspended in phosphate buffered saline. All bacterial clones were examined in parallel under exactly the same conditions. A formvar/carbon-coated grid was placed on a drop of the bacterial suspensions for 1-2 min. The grids were blotted to remove excess fluid and placed onto a drop of 0.5% phosphotungstic acid pH=4.0. Electron micrographs were made using a JOEL 1200EX electron microscope.

Acknowledgements

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Chapter 2

Antigen 43 Mediated Auto-Aggregation of *Escherichia coli* is Neutralized by Type 1 Fimbriation

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Antigen 43, the product of the *flu* gene, belongs to the group of surface displayed autotransporter proteins of Gram-negative bacteria. Ag43 is responsible for auto-aggregation and flocculation of static liquid cultures of many *Escherichia coli* strains. The expression of Ag43 has been reported to be phase variable and it has been suggested to be controlled by the product of the *oxyR* gene. Type 1 fimbriae are thin adhesive thread-like surface organelles responsible for bacterial receptor recognition and tissue colonization. Like Ag43 the expression of type 1 fimbriae is phase variable. Interestingly, previous results have suggested that expression of type 1 fimbriae and Ag43 are mutually exclusive phenotypes. In the present report we show, by use of well-defined mutants, that fimbriation in fact abolish Ag43-mediated auto-aggregation but without affecting Ag43 expression. Auto-aggregation is shown to require intercellular Ag43-Ag43 interaction and the physical presence of fimbriae on the cells seems to abrogate this. Meanwhile, the Ag43- or OxyR status does not seem to influence fimbrial expression.

Many *Escherichia coli* strains have the ability to auto-aggregate, observed as characteristic flocculation and settling of cells from liquid cultures when such are left standing. This phenomenon was first reported by Diderichsen (13) who defined a locus, *flu*, mapping at 43 min. on the *E. coli* K-12 chromosome. The *flu* locus appeared to control several surface properties. A number of strains exhibited two distinct but interconverting forms: Form 1 was characterized by large, flat, frizzy and irregular colonies and was able to aggregate in static liquid medium. The other form gave rise to smaller, glossy colonies and did not auto-aggregate in static liquid medium (13). Additionally, Diderichsen observed that certain strains with deletions in the 89 min. region were fixed in form 1. This region was later reported to harbor *oxyR* or *mor* (11, 17, 37). In separate studies it was found that the product of the *flu* locus was identical to an outer membrane protein termed antigen 43 [Ag43] (31). Ag43 was reported to consist of two equimolar protein subunits, α and β, with apparent molecular weights of 60 kDa and 53 kDa, respectively (9). The α protein is attached to the cell surface through interaction with the β component, which is an integral outer membrane protein. Ag43 expression has been proposed to be negatively controlled by OxyR and positively through dam methylation (31). Although Ag43 expression has convincingly been established to be correlated with bacterial auto-aggregation the underlying mechanism is still unknown.

Another prominent surface feature of many *E. coli* strains is type 1 fimbriae, thin, 7 nm wide and approximately 1μm long, rod-shaped surface organelles. A typical type 1 fimbriated cell has 200-500 such organelles peritrichously arranged on the

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surface. Type 1 fimbriae are widespread among members of the *Enterobacteriaceae*; they are adhesins involved in specific receptor recognition and tissue colonization (23). The expression of type 1 fimbriae is phase variable, i.e. bacterial cells with the potential to express these organelles fluctuate

TABLE 1. Bacterial strains and plasmids

Stain or plasmid	Relevant genotype, phenotype.	Reference or construction
***E. coli* K-12**		
BD1302	Δ*oxyR*, auto-aggregating	9
CC118	λ*pir* integrated on the chromosome	19
HEHA3	BD1302; Δ*fim*::*kan*	This study
HEHA10	BD1302; *flu*::*tet*	This study
MS7	PC31 Δ*fim*::*kan*	35
PC31	*fim*$^+$	21
Plasmids		
pACYC184	Cam^r, Tet^r	10
pBR322	Amp^r, Tet^r	7
pGP704	Amp^r, R6K-based origin (*pir*)	19
pHHA130	*oxyR*$^+$	A 2040 bp PCR fragment (primer 5 and 6) containing the *oxyR* gene from PC31 inserted into the *Hin*dIII site of pACYC184
pHHA145	*Sty*I site deleted	pBR322 cut with *Sty*I, made blunt with Klenow polymerase and religated
pHHA146	*flu*$^+$	A 3550 bp PCR fragment containing the *flu* gene from PC31 inserted into the *Eco*RI/*Bam*HI site of pBR322
pHHA154	*flu*$^+$	A 3550 bp PCR fragment containing the *flu* gene from PC31 inserted into the *Eco*RI/*Bam*HI site of pHHA145
pHHA159	*flu*::*tet*	A 1724 bp *Bsa*AI/*Ssp*I fragment containing the *tet* gene inserted into the (blunted) *Sty*I site of pHHA154
pHHA165	*flu*::*tet*	A 5450 bp *Eco*RI fragment containing the *flu*::*tet* construct inserted into the *Eco*RI site of pGP704
pLBJ311	Δ*fim*::*kan*	33
pMAS32	*lacUV5*::*fimA-H*	Part of the *fim* gene cluster (*fimA* to *fimH*) inserted behind the IPTG inducible *lacUV5* promoter.
pPAP5	Contains the *pap* gene cluster	25
pPKL4	Contains the *fim* gene cluster.	21
pPKL9	*fimB*$^+$	20
pPKL143	Contains the *foc* gene cluster	22

between two phenotypes, either fimbriated or bald. Phase variation of type 1 fimbriae is due to inversion of a 314 bp DNA fragment (called the *fim* switch) located immediately upstream of the structural *fim* genes (1). A promoter residing in this phase switch drives the expression of the *fim* genes (29) when the switch is in the ON orientation but not in the OFF orientation. Two recombinases, FimB and FimE (14, 20), mediates the inversion of the phase switch.

Interestingly, it was noted that the expression of Ag43 and type 1 fimbriae might be coregulated. Auto-aggregating cells were observed to be non-fimbriated, whereas non-aggregating cells were observed to be fimbriated (13). It is also noteworthy that both systems are subject to phase variation and that the phase variation frequencies seem to be similar, i.e. about 10^{-3} per cell/generation (4, 5, 31). In this communication we have explored the relationship between Ag43- and type 1 fimbriae expression and related phenotypes with a view to find possible inter-system crosstalk.

MATERIALS AND METHODS

Bacterial strains, plasmids and media. The strains and plasmids used in this study are described in Table 1. Cells were grown on solid medium or in liquid broth supplemented with the appropriate antibiotics unless otherwise stated.

DNA manipulations. Isolation of plasmid DNA was carried out using the QIAprep Spin Miniprep Kit (Qiagen). Restriction endonucleases were used according to the manufacturer's specifications (Biolabs). Chromosomal DNA purification was made using the GenomicPrep™ Cell and Tissue DNA isolation kit (Amersham Pharmacia Biotech Inc.).

PCR methodology. Polymerase chain reactions were made as previously described (35). The primers used are listed in Table 2.

Nucleotide sequencing. The nucleotide sequences of PCR products and flanking regions in the genetic constructs were determined by using the ABI PRISM BigDye Terminator cycle sequencing ready reaction kit (PE Applied biosystems). Samples were electrophoresed on a Perkin-Elmer ABI PRISM 310 Genetic Analyzer (PE Applied biosystems) as described in the manufacturer's specifications.

Construction of *flu*::*tetR* mutant. The *flu* gene was amplified by PCR from chromosomal DNA of PC31 with primer 1 and 2. The resulting fragment was inserted directly into the *Eco*RI/*Bam*HI site of pHHA145 to generate pHHA154. This plasmid was then cut with *Sty*I (1138 bp inside the *flu* gene), blunted, and a *Ssp*I/*Bsa*AI fragment from pACYC184 containing the *tetR* gene and its promoter was inserted to generate plasmid pHHA159. Plasmid pHHA159 was subsequently cut with *Eco*RI and the fragment containing the *flu*::*tetR* construct was inserted into the plasmid pGP704 and amplified in the λ*pir* carrying strain CC118. After amplification, the plasmid was transformed into the strain BD1302 and single crossover mutants were selected on 8 µg/µl Tetracycline plates. Double crossover mutants were then screened by replica plating on 8 µg/µl Tetracycline and 100 µg/µl ampicillin plates and Tet^r/Amp^s colonies were picked for further work. Correct insertion of the *flu*::*tetR* construct on the chromosome was tested by PCR using primer 3 and 4; both flanking the insertion point in *flu*. Colonies, in which PCR patterns had a shift in fragment size corresponding to the insertion, were selected and tested for loss of the auto-aggregation ability. One representative strain with this genotype was designated HEHA10 and used in this study (Fig. 1A).

Construction of *Δfim* strains. A Δ*fim* variant of BD1302 was constructed using the λ*pir* dependent plasmid pLBJ311 containing the type 1 *fim* gene cluster with a *npt* gene (Kan^r) inserted between truncated *fimB* and *fimH*, thus deleting all the *fim* genes. The insertion on the chromosome was done basically as described above and as described in (35). Correct inserts were verified by PCR and southern blotting (Fig. 1B) as previously described (34). Clones carrying the correctinsertion were designated HEHA3 and tested for loss of mannose sensitive yeast agglutination.

TABLE 2. Primers used in this study

Name	Nucleotide sequence (5´-3´)
Primer 1	CCCGCGGCCGCGATATCCTTTGTCAGTAACATGC
Primer 2	CCCGCGGCCGCGGATCCTGTGGCGTTGAAGATCCG
Primer 3	CGCTGAGCAATGACATCCG
Primer 4	AATGTCACCCTGAAGCAGG
Primer 5	GGGAAGCTTGCGGCCGCTTAGCAGGCTGGCTGGG
Primer 6	GGGAAGCTTGCGGCCGCAAAGGTGGCGGCAACAC
Primer 7	GGGAAGCTTGCGGCCGCTTAGCAGGCTGGCTGGG
Primer 8	GGGAAGCTTGCGGCCGCAAAGGTGGCGGCAACAC

Cloning of the *oxyR* gene. The *oxyR* gene was amplified by PCR from chromosomal DNA of PC31 with primer 7 and 8. The resulting fragment was inserted directly into the *Hin*dIII site of pACYC184 to generate pHHA130.

Auto-aggregation assay. Overnight cultures of the strains were adjusted to approximately the same OD_{600} and 10 ml of each culture was put into a sterile 20 ml tube. At the beginning of each experiment, all cultures were vigorously shaken for 10 seconds and 2 times 100 µl from each tube, taken approximately 1 cm from the top, were transferred to two new tubes each containing 1 ml 0.9 % NaCl and OD_{600} was measured.

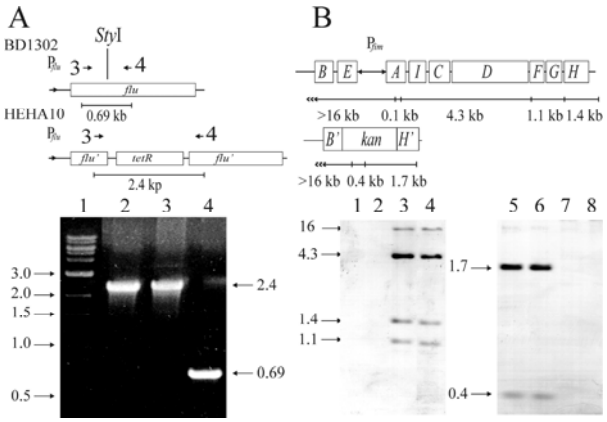

FIG. 3. (A) PCR analysis of pHHA159 (lane 2), HEHA10 (lane 3) and BD1302 (lane 4) using primers 3 and 4. Size marker (lane 1) with the sizes indicated at the left. The relevant sizes of markers and PCR bands are indicated with arrows. Above are a schematic representation of the two genetic variants and the position of the primers. (B) Southern blot hybridization using a *fim* probe (lanes 1 to 4) and a *kan* probe (lanes 5 to 8) of chromosomal DNA cut with *Pvu*II of MS7 (lanes 1 and 5), HEHA3 (lanes 2 and 6), PC31 (lanes 3 and 7) and BD1302 (lanes 4 and 8). Fragment sizes (in kb) are indicated by arrows to the left. Above are schematic representations of the two genetic variants and the positions of the *Pvu*II sites in these.

Detection of type 1 fimbriae. The capacity of bacteria to express a D-mannose-binding phenotype was assayed by their ability to agglutinate yeast cells on glass slides. Aliquots of liquid cultures grown at an optical density of 4.0 and 5% (w/v) suspension of yeast cells were mixed and the time until agglutination occurred was measured.

Immunofluorescence microscopy. Surface presentation of type 1 fimbriae and Ag43, respectively, was assessed by immunofluorescence microscopy employing a monoclonal antibody directed against FimA (35) or a polyclonal serum that recognizes the α-subunit of Ag43 (a kind gift from Peter Owen). Cell fixation, immunolabeling and microscopy was carried out as previously described (32) employing a FITC-labeled secondary antibody.

RESULTS

The *flu* locus and Ag43. The published N-terminal sequences of the α and β fragments of Ag43 (31) are contained within an open reading frame of 1039 codons located at a map position at 43.6 min. of the *E. coli* K-12 chromosome (3) and therefore coincides with the *flu* locus. A strong consensus ribosomal binding site precedes the start codon suggesting a theoretical molecular weight of 106.9 kDa for the primary product of the *flu* gene. N-terminal sequencing indicates that the mature α fragment starts another 52 amino acids into this sequence. This would suggest a mature Ag43 protein of 987 amino acids (101.6 kDa). The reported N-terminal sequence of the β fragment (31) indicates that this protein is further processed into an α fragment of 499 a.a. (49.8 kDa) and a β fragment of 488 a.a. (51.5 kDa); in agreement with the reported apparent molecular weights of 50-60 kDa (α-fragment) and 53 kDa (β-fragment) given in the literature (9).

Cloning of the *flu* gene and construction of a defined knock-out mutant strain. Two primers were made with sequences corresponding to positions 322 bp upstream and 76 bp downstream of ORF1039, respectively, and used to amplify the *flu* gene from *E. coli* K-12 strain PC31. The 3.5 kb PCR fragment was cloned into pBR322 resulting in plasmid pHHA146. Subsequently, a *tet* cassette was inserted into the *flu* gene and the *flu::tet* construct inserted into the suicide vector pGP704 to result in pHHA165. *E. coli* strain BD1302 has a large chromosomal deletion in the 89 min. region encompassing the *oxyR* gene (13, 37) resulting in constitutive expression of Ag43. Plasmid pHHA165 was used to make a defined knock-out mutant of the *flu* gene in this strain by homologous recombination with the *flu::tet* cassette resulting in strain HEHA10 (Fig. 1A). When liquid cultures of strain BD1302 are left standing the cells readily

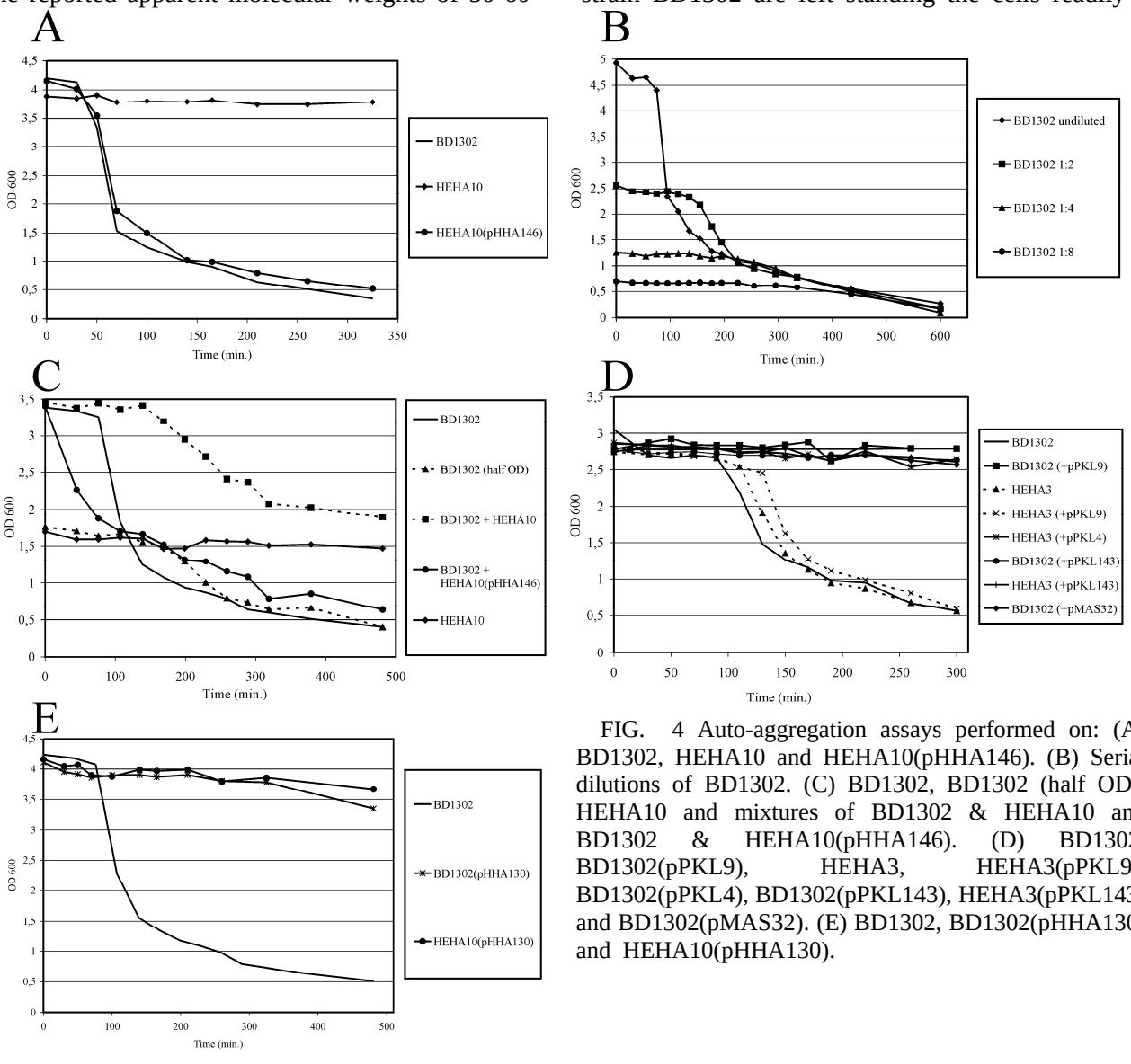

FIG. 4 Auto-aggregation assays performed on: (A) BD1302, HEHA10 and HEHA10(pHHA146). (B) Serial dilutions of BD1302. (C) BD1302, BD1302 (half OD), HEHA10 and mixtures of BD1302 & HEHA10 and BD1302 & HEHA10(pHHA146). (D) BD1302, BD1302(pPKL9), HEHA3, HEHA3(pPKL9), BD1302(pPKL4), BD1302(pPKL143), HEHA3(pPKL143) and BD1302(pMAS32). (E) BD1302, BD1302(pHHA130) and HEHA10(pHHA130).

auto-aggregate and settle (Fig. 2A). In contrast, cells from liquid cultures of the isogenic *flu::tet* strain HEHA10 stay in solution under similar conditions (Fig. 2A). Meanwhile, when the Ag43-expressing plasmid pHHA146 was introduced into HEHA10 an auto-aggregating phenotype identical to that of BD1302 could be re-established (Fig. 2A). In order to gain further insight into Ag43 expression in these strains immunofluorescence microscopy with specific anti-Ag43 serum was performed (Fig. 3B and 3D). It became clear from these results that the auto-aggregating phenotype was concomitant with the ability to express Ag43 and that Ag43 must be directly responsible for this property.

Ag43-mediated auto-aggregation kinetics. To gain more insight to the auto-aggregation phenomenon, we investigated the kinetics of the Ag43-mediated auto-aggregation, i.e. whether this was dependent on cell density. For this purpose the settling profiles of different concentrations of BD1302 were analyzed. It was apparent (Fig. 2B) that BD1302 cells settled regardless of the cell density used, i.e. virtually all cells were settled by the end of the experiment. Nevertheless, the time that passed before aggregation was initiated was highly dependent on the cell density of the initial suspension. Roughly, when the cell density was halved, the initiation point was doubled. This is in good agreement with a model following first order kinetics, where the chance of two bacteria colliding at a given time interval is proportional to the cell density.

Ag43-Ag43 interaction is responsible for bacterial auto-aggregation. In order to investigate whether Ag43 mediated auto-aggregation was caused by Ag43-Ag43 interaction between aggregating cells or, alternatively, whether Ag43 on one cell interacted with a non-Ag43 target on another cell, the following experiments were carried out. A model featuring Ag43-Ag43 interaction as responsible for auto-aggregation would suggest that mixing equal aliquots of isogenic $Ag43^+$ and $Ag43^-$ cells would result in precipitation of only half of the cells, i.e. $Ag43^-$ cells would not participate. This was exactly what was observed in settling experiments of equal amounts of BD1302 and HEHA10 cells (Fig 2C). Furthermore, careful sampling and plating of cells revealed that virtually all cells remaining in suspension were HEHA10 and precipitated cells were BD1302. Introduction of plasmid pHHA146 into HEHA10 and mixing such cells with equal amounts of BD1302 cells caused settling identical to a monoculture of BD1302 cells (Fig. 2C). This strongly suggests that intercellular Ag43-Ag43 interaction is responsible for the auto-aggregation phenomenon.

Ag43-mediated auto-aggregation is abolished by fimbriation. As previously noted several observations have hinted that expression of Ag43 and type 1 fimbriation might be mutually exclusive phenotypes and that the responsible genes might be reciprocally regulated (13). The support for this tenet was based on the observation that cells from glossy colonies are $Ag43^-$, do not form auto-aggregates and agglutinate yeast cells in a D-mannose sensitive manner (indicative of type 1 fimbriation) whereas frizzy colonies are $Ag43^+$, auto-aggregate and does not agglutinate yeast cells. *E. coli* strain BD1302 is the classic Ag43 reference strain. In order to investigate potential inter-system coregulation of Ag43 and type 1 fimbriae derivatives of BD1302 were made which were forced to produce type 1 fimbriae. This was done in two ways, either by introduction of a high copy number plasmid, pPKL4, encoding the *fim* gene cluster or by activating the resident *fim* gene cluster of BD1302 by introduction of a plasmid, pPKL9, encoding the *fimB* recombinase. Strain BD1302 readily auto-aggregates, however, both BD1302(pPKL4) and BD1302(pPKL9) had lost this faculty (Fig. 2D). In order to examine, whether FimB caused this effect by activation of the resident *fim* gene cluster with ensuing fimbriation or whether abolishing of auto-aggregation was due to a yet unknown effect of the recombinase, a derivative of BD1302 was made, HEHA3, in which the *fim* gene cluster was deleted (cf. Materials and Methods). Both HEHA3 and HEHA3(pPKL9) auto-aggregated exactly like BD1302 (Fig. 2D). However, a control strain HEHA3(pPKL4) did not. As a further control we introduced a plasmid (pMAS32), containing a *fim* gene cluster in which the recombinase genes and phase switch had been replaced by a *lacUV5* promoter, into BD1302. BD1302(pMAS32) cells did not auto-aggregate (Fig. 2D). These experiments indicated that it was the physical presence of fimbriae on the cells that abolished auto-aggregation. To further examine this hypothesis plasmids harboring the genetically distinct *foc* gene cluster (pPKL143) and *pap* gene cluster (pPAP5) encoding respectively F1C and P-fimbriae, were introduced into BD1302 and HEHA3. All four strains, BD1302(pPKL143), BD1302(pPAP5), HEHA3(pPKL143) and HEHA3(pPAP5) had lost their ability to auto-aggregate (see for example Fig. 2D).

Ag43 expression is not affected by fimbriation. The above results could be interpreted in two ways, either the physical presence of the fimbriae on the bacterial surface prevented the Ag43 expressing bacteria from establishing the required contact for Ag43-Ag43 interaction to take place

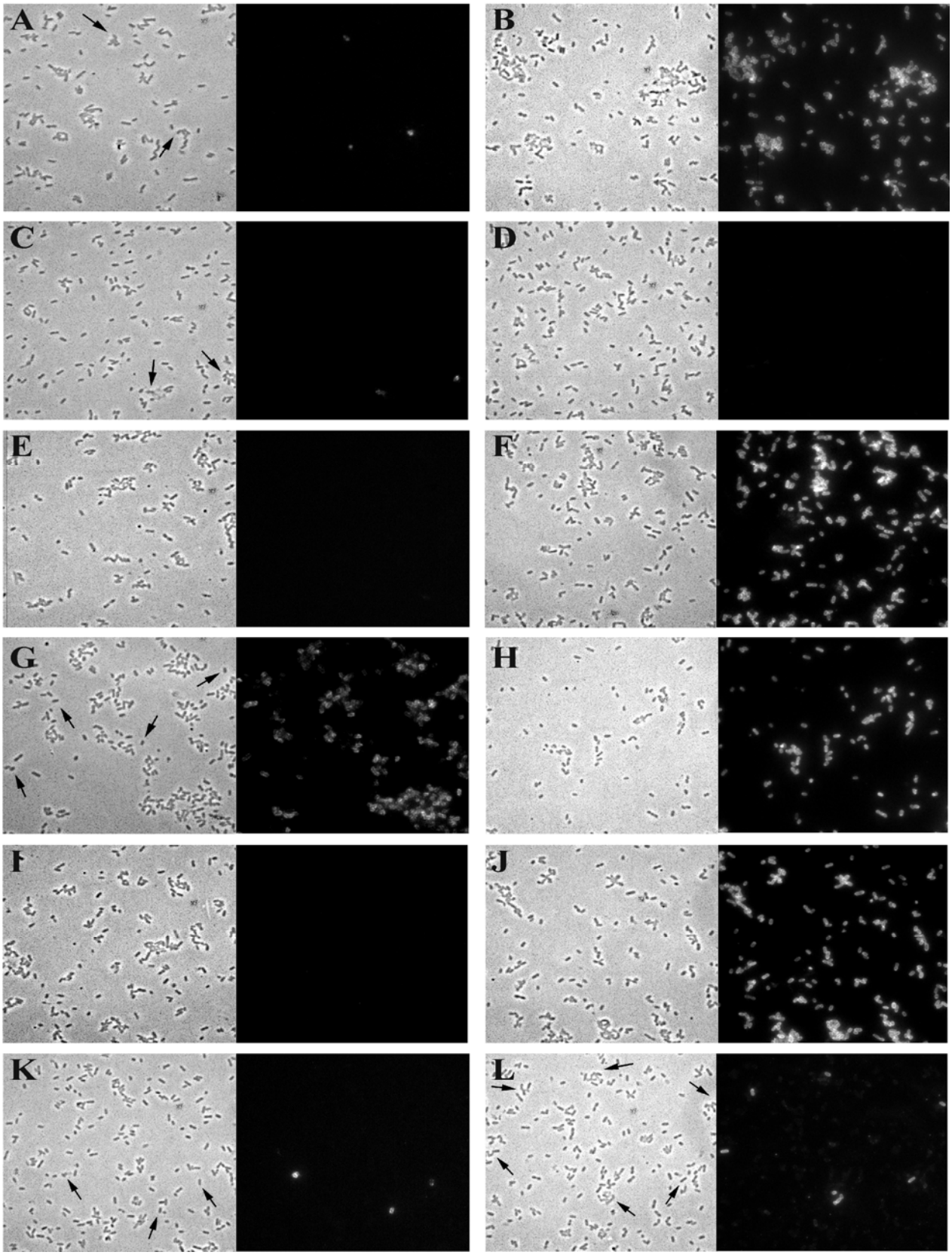

FIG. 3. Phase Contrast Microscopy (PCM) and Fluorescence Microcopy (FM) of *E. coli* K-12 hosts. To detect presence of type 1 fimbriae on the surface of the bacteria (left side of the figure), a monoclonal antibody directed against FimA was used, and this was detected by a FITC labeled rabbit anti mouse serum. To detect the presence of Ag43 on the surface of the bacteria (right side of the figure), a FITC labeled pig anti rabbit serum was used. The following strains were tested. (A) and (B) BD1302, (C) and (D) HEHA10, (E) and (F) HEHA3, (G) and (H) BD1302(pPKL9), (I) and (J) HEHA3(pPKL9) and (K) and (L) BD1302(pHHA130). Arrows indicate cells expressing a different phenotype than the majority.

thus abrogating auto-aggregation, or, alternatively, that fimbrial expression somehow excluded Ag43 expression. To examine these possibilities selected strains were submitted to immunofluorescence microscopy employing specific antisera raised against type 1 fimbriae or Ag43. Not surprisingly, BD1302 cells were observed to react strongly with Ag43-specific antibodies (Fig. 3B); also a small percentage of the cells reacted with fimbriae-specific serum (Fig. 3A). Deletion of the Ag43-encoding gene on the chromosome, *viz.* strain HEHA10, did not change the percentage of fimbriated cells (Fig. 3C) but, not unexpectedly, resulted in the disappearance of Ag43 from the cells (Fig. 3D). Likewise HEHA3 cells in which the *fim* gene cluster was deleted showed no reaction with anti-fimbriae serum, whereas the Ag43 level was identical to the BD1302 parent strain (Fig. 3E and 3F). Introduction of plasmid pPKL9 into BD1302 resulted in a population where virtually all cells expressed both fimbriae and Ag43 (Fig. 3G and 3H). On the contrary introduction of plasmid pPKL9 into strain HEHA3 (*Δfim*) did not affect Ag43 production, i.e. it was indistinguishable from that of the BD1302 parent, whereas, as expected no cells produced fimbriae (Fig. 3I and 3J). In the light of these results we therefore concluded that Ag43 production is unaffected by the level of fimbriation, and that fimbrial neutralization of Ag43-mediated auto-aggregation seems to be a physical rather than a co-regulation phenomenon.

Influence of *oxyR*. Strain BD1302 has a large deletion in the 89 min. region encompassing the *oxyR* locus and a number of flanking genes. It is a constitutive Ag43 producer and this phenotype has, with some justification, been assumed to be due to the lack of OxyR in the cells, although this has never been stringently proven. In order to do this and to investigate the influence of OxyR on fimbrial expression the *oxyR* locus in *E. coli* strain PC31 was amplified and cloned into plasmid pACYC184 resulting in plasmid pHHA130 (cf. Materials and Methods). Introduction of plasmid pHHA130 into BD1302 virtually abolished the capacity to auto-aggregate (Fig. 2E). In keeping with this result, immunofluorescence microscopy revealed a dramatic decline in the number of Ag43 producing cells (Fig. 3L). However, the fraction of fimbriated cells seemed to be the same as in the parent strain (Fig. 3K). This would suggest that OxyR does indeed repress Ag43 production but seems to have no influence on fimbriation.

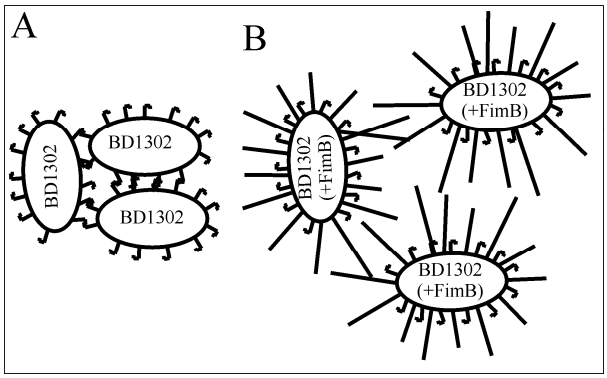

FIG. 4. Schematic model for relationship between fimbriae production and auto-aggregation.

DISCUSSION

Ag43 belongs to the growing family of autotransporter proteins from Gram-negative bacteria. The entire sequence information required to mediate transport and secretion through the outer membrane is contained within the protein itself and has been provisionally referred to as type IV secretion (18). Members of the autotransporter family include important or putative virulence factors in many pathogens. Some, like the IgA1 protease of *Neisseria gonorrhoeae*, are proteases (26), others, like the AIDA-I protein of diarrhoeagenic *E. coli*, are adhesins (2). Type 1 fimbriae are adhesins associated with many *E. coli* strains, and have recently been shown to be critical for the ability of *E. coli* to colonize the urinary tract (12, 24, 28). Assembly and surface presentation of type 1 fimbriae follows a different pathway than Ag43 and require, like other fimbriae, chaperone- and usher-assisted assembly to cross the outer membrane.

Evidence accumulating in the literature have pointed to possible coregulation of type 1 fimbriae and Ag43 and prompted us to examine this observation in greater detail. Work on Ag43 and on potential Ag43-type 1 fimbrial crosstalk has been hampered by the lack of defined mutant strains. In this study we have focussed on the reference strain BD1302, originally characterized as a constitutive expressor of Ag43 (13). Suspensions of BD1302 cells readily auto-aggregate and settle, however, replacement of the *flu* gene in BD1302 with a *flu::tet* cassette abolished this property (Fig. 2A). Complementation of the mutant with a plasmid encoding the *flu* gene reestablished the parental phenotype. Having established Ag43 as the causative agent of auto-aggregation in BD1302 we proceeded to investigate the underlying mechanism for the auto-aggregation, i.e. whether it was Ag43-Ag43 based or whether Ag43 recognized some other feature on the surface of *E. coli*. It turned out that Ag43-Ag43 interaction indeed seemed to be responsible for cell aggregation because in settling experiments with mixtures of the isogenic strains BD1302 and HEHA10 (*flu::tet*) the latter did not participate in the aggregation process. Also, the

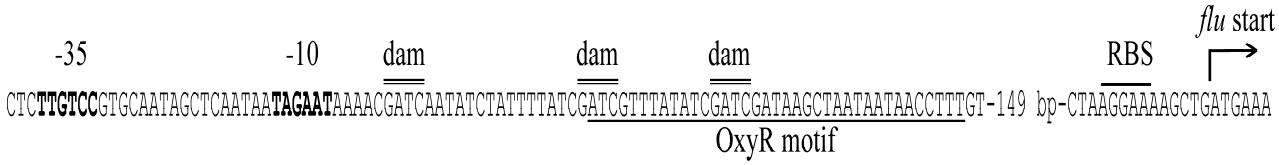

FIG. 5. Schematic presentation of the promoter region of the *flu* gene showing the location of the suggested promoter, dam methylation sites, suggested OxyR binding site, ribosome binding site (RBS) and translation initiation site.

results from the settling experiments suggested that Ag43-mediated auto-aggregation followed first order kinetics. BD1302 has a functional *fim* gene cluster but only a few percent of the cells at a given time were actually seen to express the organelles due to phase variation (Fig. 3). We have previously observed that introduction of the *fimB* recombinase gene on a high copy number plasmid results in a population where virtually all cells are fimbriated (27). This was also seen with BD1302(pPKL9) cells (Fig. 3). Introduction of a plasmid encoding the entire *fim* gene cluster had the same effect. In contrast to the BD1302 parent such bacteria were unable to auto-aggregate (Fig. 2D). The abolishment of auto-aggregation could be due to crosstalk between the Ag43 and type 1 fimbrial systems, however, neutralization of auto-aggregation was not seen when pPKL9 was introduced into a derivative of BD1302 where the chromosomal *fim* genes had been deleted, i.e. in HEHA3. Furthermore, it was found, by immunofluorescence microscopy, that Ag43 production was not affected by concomitant type 1 fimbriation. This suggested that the physical presence of fimbriae on the cell surface negated the Ag43-Ag43 contact between cells and thereby prevented the auto-aggregation phenotype (Fig. 4). This conclusion was further corroborated by the fact that introduction of plasmids encoding the *foc* and *pap* gene clusters, respectively, into BD1302 also neutralized auto-aggregation. In this regard it should be emphasized that the expression control systems of F1C and P-fimbriae differ fundamentally from that of type 1 fimbriae (6, 8, 30, 33).

Ag43 expression has been suggested to be negatively controlled by OxyR and positively by dam methylation. Henderson *et al.* (16) proposed an elegant model for regulation of Ag43 expression in which OxyR acts as a repressor by binding to unmethylated dam sites in the regulatory region of the *flu* gene. Also, following this model, methylation prevents OxyR binding. Analysis of the region upstream of the *flu* gene identified a potential sigma-70 promoter located around 240 bp upstream of the start codon. Three GATC-sites overlapping with a motif similar to the proposed consensus sequence for OxyR-DNA binding (36) were found in this intergenic region (Fig. 5). It has not stringently been shown that OxyR regulates Ag43 expression. For this purpose we amplified the *oxyR* gene from *E. coli* K-12 strain PC31 and cloned it on a pACYC184 vector (plasmid pHHA130). Introduction of plasmid pHHA130 into BD1302 virtually abrogated the auto-aggregation phenotype (Fig. 2) and the number of Ag43 producing cells dropped dramatically (Fig. 3 K and L). The residual Ag43 producing cells could be accounted for by inability of OxyR to bind to methylated GATC sites in accordance with the model of Henderson et al. (16). Interestingly, the fraction of cells expressing type 1 fimbriae was observed to be the same in BD1302 and BD1302(pHHA130) (Fig. 3). It therefore seems that neither the Ag43 status nor the OxyR status of the cells affects type 1 fimbriation.

The biological *raison d'être* for Ag43 production is somewhat controversial. Ag43 has been reported to confer low level of adhesion to certain mammalian cells (31) but not in a manner that seems compatible with its widespread occurrence in *E. coli* strains. Since Ag43 is a self-recognizing protein which causes bacterial aggregation this faculty might have a function in a mammalian host. However, the binding strength of Ag43-Ag43 mediated cell interaction is very low compared to the binding mediated by for example fimbrial adhesins, and Ag43-auto-aggregated cells are easily dispersed. Clearly auto-aggregating bacterial clumps may confer increased survival to individual bacteria under different conditions in many environments and perhaps the mission of Ag43 is outside a mammalian host. It might be that the role of Ag43 indeed is to cause auto-aggregation of cells with ensuing settling under static liquid conditions. In this respect it is interesting to speculate that when *E. coli* is shed by defecating animals in stagnant pools of water it might be of advantage for the bacteria to become bottom dwellers in order to avoid U.V. radiation in the surface layer.

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Chapter 3

The Role of Antigen 43 and Type 1 Fimbriation in *Escherichia coli* Colony Morphology.

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Preliminary note

Due to the phase variation of Antigen 43, colonies with cells expressing this protein have a different morphology than colonies containing cells, which are not expressing Antigen 43. These, on the other hand, have been reported to express type 1 fimbriae. The former colony morphology form, called *frizzy* or Form 1, is large, flat, has a jagged edge and a rough surface. The latter colony morphology form, called *glossy* or Form 2, is small, high, has a circular edge and a smooth surface. Here it is shown that there in fact exists a third colony morphology type, called Form 3, which is large, flat, has a jagged edge and a smooth surface. This type is shown, by immunofluorescence microscopy employing specific antibodies directed against either FimA or Ag43, to consist of cells, which neither produce type 1 fimbriae or Antigen 43. By constructing defined *flu* and *fim* mutants it is concluded, that the rough surface appearance of Form 1 colonies, but not the size variation, can be accounted for by Antigen 43 production. The size variation is suggested to be caused by phase variation of type 1 fimbriae on the cells and to be connected to an insertion in the *fimE* gene.

Traditionally, the colony morphology of *E. coli* is separated into either rough (R) or smooth (S) form. The two forms are readily distinguished, as the colonies of the former are smooth, high and circular and colonies of the latter are rough, flat and irregular. Most *E. coli* K-12 strains are rough due to insertion of an IS5 element in the *rfb* gene cluster controlling the O antigen biosynthesis in the lipopolysaccharides of the outer membrane (Liu and Reeves, 1994). Only rarely the K-12 strains are able to revert to the smooth form, as this requires the integrity of the *rfb* gene cluster to be restored. Therefore, frequent switching between the two forms is virtually impossible.

Diderichsen (1980) showed and partially identified another kind of mechanism controlling the colony morphology of K-12 strains. The observed phenotypes were somewhat similar to the ones seen for rough and smooth strains (Fig. 1), but the strains were frequently switching between the two. To distinguish this from the rough and smooth phenotypes related to lipopolysaccharides, the rough, flat, irregular and auto-aggregating variant was designated *frizzy* (or Form 1 by Warne *et al*., 1990) and the smooth, high, circular and non-aggregating *glossy* (or Form 2). The locus responsible for this change in phenotype was mapped to a position between 43.5 and 43.7 minutes on the *E. coli* chromosome and named *flu*, where the nonfluffing cells by default were assigned *flu*$^+$ and the fluffing were assigned *flu*.

In an independent study the protein responsible for the auto-aggregating phenotype was identified as a bipartite protein antigen associated with the outer membrane and named Antigen 43 (Ag43)

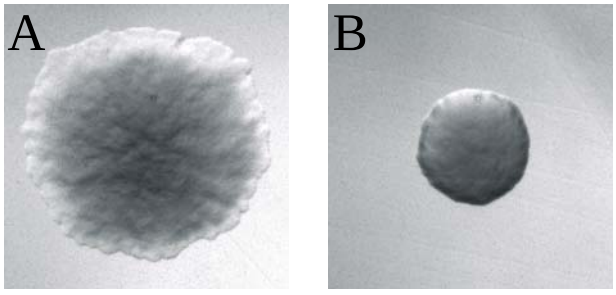

Fig. 5. Representative examples of (A) a frizzy colony (BD1512) and (B) a glossy colony (BD1511).

(Owen, 1983). It was found to consist of two

TABLE 1. Bacterial strains and plasmids

Stain or plasmid	Relevant genotype, phenotype.	Reference or construction
E. coli K-12		
BD1302	Δ*oxyR*, auto-aggregating	Diderichsen, 1980
BD1511	*fimE* $^{-}$, non-aggregating, glossy colonies	Diderichsen, 1980
BD1512	*fimE* $^{-}$, auto-aggregating, frizzy colonies	Diderichsen, 1980
CC118	λ*pir* integrated on the chromosome	Herrero, 1990
CSH50	*fimE*::IS*1*	Blomfield *et al*., 1991
HEHA4	BD1511/BD1512; Δ*fim*::*kan*	This study
HEHA6	BD1511/BD1512; *fimE*$^{+}$	This study
HEHA9	BD11511/BD1512; *ΔoxyR*::*Ω*(Sp^{R})	This study
HEHA11	BD1511/BD1512; *ag43*::*tet*	This study
N9716	GC4468; *ΔoxyR*::*Ω*(Sp^{R})	Storz, unpublished
PC31		Klemm *et al*., 1985
Plasmids		
pACYC184	Cam^{r}, Tet^{r}	Chang and Choen, 1978.
pBR322	Amp^{r}, Tet^{r}	Bolivar, 1978
pGP704	Amp^{r}, R6K-based origin (*pir*)	Herrero, 1990
pHHA130	Cam^{r}, *oxyR*$^{+}$	A 2040 bp PCR fragment (primer 5 and 6) containing the *oxyR* gene from PC31 inserted into the *Hin*dIII site of pACYC184
pHHA145	Amp^{r}, Tet^{r}, *Sty*I site deleted	pBR322 cut with *Sty*I, made blunt with Klenow polymerase and religated
pHHA146	Amp^{r},	A 3522 bp PCR fragment (primer 1 and 2) containing the *ag43* gene from PC31, inserted into *Eco*RI/*Bam*HI site of pBR322
pHHA154	Amp^{r},	A 3550 bp PCR fragment containing the *ag43* gene from PC31 inserted into the *Eco*RV site of pHHA145
pHHA159	Amp^{r}, Tet^{r}	A 1724 bp *Bsa*AI/*Ssp*I fragment containing the *tet* gene inserted into the (blunted) *Sty*I site of pHHA154
pHHA161	Cam^{r} *fimE*$^{+}$-*fimC*$^{+}$	A 5492 bp *Hin*dIII/*Eco*RI fragment containing the *fimE* to the *fimC* gene inserted in the same sites of pMAK700oriT
pHHA165	Amp^{r}, Tet^{r}, *ag43*::*tet*	A 5450 bp *Eco*RI fragment containing the *ag43*::*tet* construct inserted into the *Eco*RI site of pGP704
pLBJ311	Amp^{r},	Schembri *et al*., 1996
pMAK700oriT	Cam^{r}, Ts origin	Chakraborty, unpublished
pMAS32	Amp^{r}, *lacUV5*::*fimA-H*	Part of the *fim* gene cluster (*fimA* to *fimH*) inserted behind the IPTG inducible *lacUV5* promoter.
pPKL4	Amp^{r}, contains the *fim* gene cluster.	Klemm *et al*., 1985

polypeptides termed α and β (M_ws of 60 and 53 kDa, respectively) in a 1:1 ratio (Owen *et al*., 1987). This protein complex shows relatively high (31.2 %) identity (Henderson and Owen, 1999) to AIDA-I, a 100 kD outer membrane adhesin associated with diffuse adhering of enteropathogenic *E. coli* to small bowel mucosa (Benz and Schmidt, 1989). Unfortunately, a rather confusing terminology relationship existed between Ag43 and *flu*. In reality Ag43 in fact turned out to be the product of the *flu* gene. This means, that a mutant with a mutated *flu* gene is unable to auto-aggregate and is Ag43^{-} and transcription of the *flu* gene results in Ag43 expression Ag43^{+} (Owen *et al*., 1996; Hasman *et al*., Chapter 2). Diderichsen (1980) also in part identified the regulatory mechanism controlling the phase variable expression of Ag43, as a mutant (strain BD1302) with a deletion in the 89 minutes region was locked in the *frizzy* (Ag43^{+}) phenotype. This gene was later identified as the *oxyR* (*mor*) gene (Warne *et al*., 1990; Henderson and Owen, 1997). OxyR is homologous to the LysR family of bacterial proteins and like these, it works as an activator of a regulon of genes and additionally act as an autorepressor (Storz *et al*., 1990; Storz and Altuvia, 1994). OxyR is normally involved in protection against oxidative stress as it activates a regulon of peroxide-inducible genes (Kullik *et al*., 1995a & 1995b), but it also represses the *flu* gene (Henderson and Owen, 1999; Hasman *et al*., Chapter 2). Apart from OxyR, also the Dam protein, responsible for methylating GATC sites of DNA, is involved in the expression of Ag43. A *dam* mutant is, contrary to an *oxyR* mutant, unable to express Ag43, whereas a double, *dam*, *oxyR*, mutant is a constitutive Ag43 expresser (Henderson and Owen, 1999).

Another surface feature of *E coli* subject to phase variation is type 1 fimbriae encoded by the *fim* gene cluster, which confer mannose sensitive adhesion and agglutination of yeast cells (Reviewed by Krogfelt and Klemm, 1994). The phase switching mechanism of these rod-like

appendages is quite different from the one described for Ag43. The *fim* gene cluster contains a promoter on a 314 bp invertible DNA fragment called the *fim* switch (Abraham *et al*., 1985; Freitag *et al*., 1985). Upstream of this fragment are the genes encoding two recombinases, *fimB* and *fimE* (Klemm, 1986, Pallesen, 1989). Under normal aerated growth conditions, FimB is able to catalyze inversion of the switch in both directions (Klemm, 1986, McClain, 1991), whereas FimE only catalyze ON-to-OFF switching (Blomfield *et al*., 1991 and 1993), which leads to non-fimbriated cells.

According to observations by Diderichsen (1980), the auto-aggregating and the fimbriated phenotype seemed to be mutually exclusive, that is, $Ag43^-$ cells were Fim^+ and $Ag43^+$ cells were Fim^-, at least in the strain set used (BD1511/BD1512). This suggested that type 1 fimbriae and Ag43 are in opposite phase, *viz.* $Ag43^+$ cells form frizzy colonies and does not agglutinate yeast cells and $Ag43^-$ cells form glossy colonies and perform mannose sensitive yeast agglutination. A correlation between fimbriation and colony morphology has later been reported in an independent study involving the *E. coli* K-12 strain CSH50. This strain carries an IS*1* insertion in the *fimE* gene (Blomfield *et al*, 1991). *E. coli* K-12 strain MG1655 contains an intact *fim* gene cluster and makes only large flat colonies. Replacement of the *fimE* gene in MG1655 with the truncated *fimE* gene from CSH50 causes this to make both large and small colonies similar to CSH50 (Blomfield *et al*, 1991). However, the Ag43 status was not investigated of the strains. From these observations it is clear, that the data concerning the origin of phase variation of colony morphology are somewhat controversial, some refer the phenomenon to antigen 43 expression and others to type 1 fimbriation. On this background the two phase variable systems were examined with regard to colony morphology.

MATERIAL AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are described in Table 1. Cells were grown on solid medium or in liquid broth supplemented with the appropriate antibiotics unless otherwise stated.

DNA manipulations. Isolation of plasmid DNA was carried out using the QIAprep Spin Miniprep Kit (Qiagen). Restriction endonucleases were used according to the manufacturer's specifications (Biolabs). Chromosomal DNA purification was made using the GenomicPrep™ Cell and Tissue DNA isolation kit (Amersham Pharmacia Biotech Inc.).

PCR methodology. Polymerase chain reactions were made as previously described (Stentebjerg-Olesen et al., 1997). The primers used are listed in table 2.

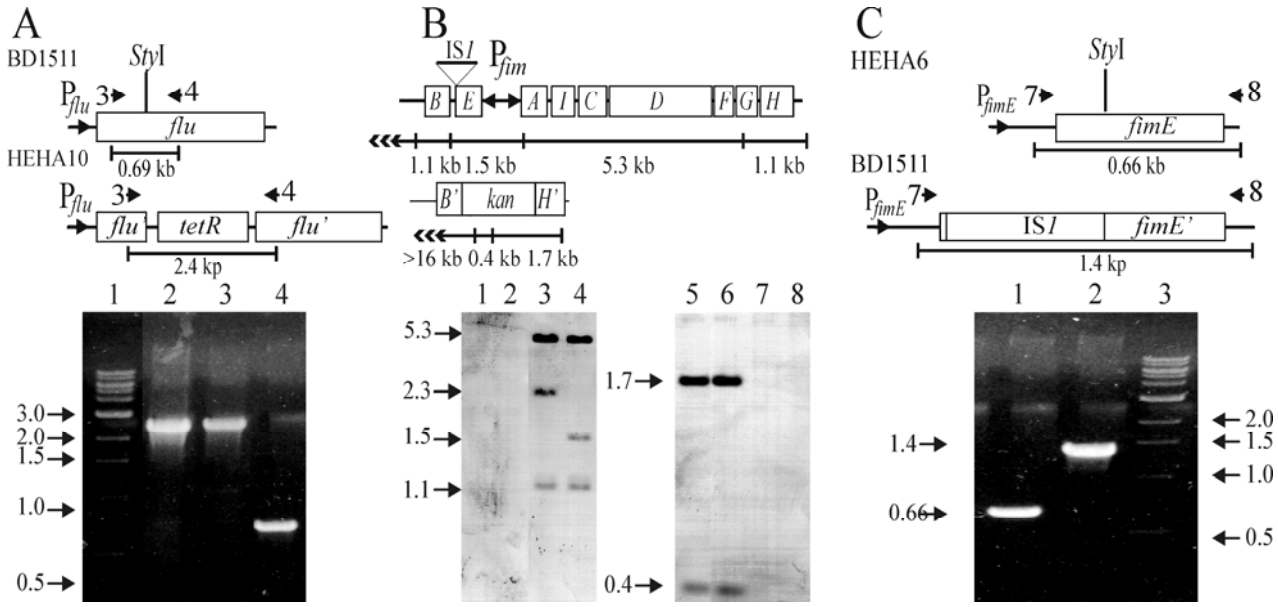

Fig. 6. (A) PCR analysis of pHHA159 (lane 2), HEHA11 (lane 3) and BD1511 (lane 4) using primers 3 and 4. Size marker (lane 1) with the sizes indicated at the left. The relevant sizes of markers and PCR bands are indicated with arrows. Above are a schematic representation of the two genetic variants and the position of the primers. (B) Southern blot hybridization using a *fim* probe (lanes 1 to 4) and a *kan* probe (lanes 5 to 8) of chromosomal DNA cut with *Pvu*II of MS7 (lanes 1 and 5), HEHA4 (lanes 2 and 6), BD1511 (lanes 3 and 7) and PC31 (lanes 4 and 8). Fragment sizes (in kb) are indicated by arrows to the left. Above are schematic representations of the wild type *fim* gene cluster with the IS*1* element indicated and the *Δfim::kan* construct. In both cases, the positions of the *Pvu*II sites in these are indicated. Note the shift of the 1.5 kb fragment in lane 4 to the 2.3 kb fragment in lane 3 illustrating the IS*1* element. (C) PCR analysis of the *fimE* gene in HEHA6 (lane 1) and BD1511 by using the primers 7 and 8. Size marker (lane 3) with the sizes (in kb) indicated at the left. The relevant sizes of markers and PCR bands are indicated with arrows.

TABLE 2. Primers used in this study

Name	Nucleotide sequence (5´-3´)
Primer 1	CCCGCGGCCGCGATATCCTTTGTCAGTAACATGC
Primer 2	CCCGCGGCCGCGGATCCTGTGGCGTTGAAGATCCG
Primer 3	CGCTGAGCAATGACATCCG
Primer 4	AATGTCACCCTGAAGCAGG
Primer 5	GGGAAGCTTGCGGCCGCTTAGCAGGCTGGCTGGG
Primer 6	GGGAAGCTTGCGGCCGCAAAGGTGGCGGCAACAC
Primer 7	GGGAAGCTTGCGGCCGCTTAGCAGGCTGGCTGGG
Primer 8	GGGAAGCTTGCGGCCGCAAAGGTGGCGGCAACAC

Nucleotide sequencing. The nucleotide sequences of PCR products and flanking regions in the genetic constructs were determined by using the ABI PRISMTM BigDye Terminator cycle sequencing ready reaction kit (PE Applied biosystems). Samples were electrophoresed on a Perkin-Elmer ABI PRISM 310 Genetic Analyzer (PE Applied biosystems) as described in the manufacturer's specifications.

Detection of type 1 fimbriae. The capacity of bacteria to express a D-mannose-binding phenotype was assayed by their ability to agglutinate yeast cells on glass slides. Aliquots of liquid cultures grown at an optical density of 4.0 and 5% (w/v) suspension of yeast cells were mixed and the time until agglutination occurred was measured.

Construction of the *ag43::tet* mutants. The *flu* gene was amplified by PCR from chromosomal DNA of PC31 with primer 1 and 2. The resulting fragment was inserted directly into the *Eco*RI/*Bam*HI site of pHHA145 to generate pHHA154. This plasmid was then cut with *Sty*I (1138 bp inside the *flu* gene), blunted, and a *Ssp*I/*Bsa*AI fragment from pACYC184 containing the *tetR* gene and its promoter was inserted to generate plasmid pHHA159. Plasmid pHHA159 was subsequently cut with *Eco*RI and the fragment containing the *flu*::*tetR* construct was inserted into the plasmid pGP704 and amplified in the λ*pir* carrying strain CC118. After amplification, the plasmid was transformed into the to the strain set BD1511/BD1512 and single crossover mutants were selected on 8 µg/µl Tetracycline plates. Double crossover mutants were then screened by replica plating on 8 µg/µl Tetracycline and 100 µg/µl ampicillin plates and Tet^r/Amp^s colonies were picked for further work. Correct insertion of the *flu*::*tetR* construct on the chromosome was tested by PCR using primer 3 and 4; both flanking the insertion point of the *flu* gene. Colonies, in which PCR patterns had a shift in fragment size corresponding to the insertion, were selected and tested for loss of the auto-aggregation ability. One representative strain with this genotype was designated HEHA11 and used in this study (Fig. 2A).

Construction of the* oxyR::Ω(Sp^R) *mutant. *A P1 phage lysate was made from the* oxyR::Ω(Sp^R) *strain N9716 and used to transfect the strain set BD1511/BD1512 as described by Miller (1992). Double crossover mutants were selected on LB plates containing spectinomycin (15 µg/ml) and correct inserts were tested by PCR with primers 5 and 6 (Table 2) annealing to regions flanking the* oxyR *gene). A clone with the same PCR fragment size as N9716 was selected, named HEHA9 and used in this study.*

Construction of the *Δfim* mutants. A *Δfim* variant of the BD1511/BD1512 strain set was constructed using the λ*pir* dependent plasmid pLBJ311 containing the type 1 *fim* gene cluster with a *npt* gene (Kan^r) inserted between truncated *fimB* and *fimH*, thus deleting all the *fim* genes. The insertion on the chromosome was done basically as described above and as described in (Stentebjerg-Olesen *et al*., 1997). Correct inserts were verified by PCR and southern blotting (Fig. 2B) as previously described (Schembri *et al*., 1996). A clone carrying the correct insertion was designated HEHA4 and tested for loss of mannose sensitive yeast agglutination.

Allelic exchange procedure of the *fimE* gene of BD1511. In order to restore the *fimE* gene in the strain set BD1511/BD1512, part of the *fim* gene cluster (downstream of *fimB* to the middle of *fimD*) was inserted into the integration vector pMAK700oriT by cutting pPKL4 with *Hin*dIII and *Eco*RI and inserting the *fim* fragment into the same sites in pMAK700oriT. This plasmid carries a temperature sensitive origin of replication, which only permits it to replicate at temperatures below 30 °C. The resulting plasmid was named pHHA161, transformed into BD1511 and allowed to grow over night at 30 °C on 17 µg/µl chloramphenicol (Cam) plates. After re-streaking single colonies at 30 °C for two days, single colonies were streaked out on 17 µg/µl Cam plates and placed at 42 °C to select for *recA*-mediated single crossover events. Cam^r colonies were then grown in liquid LB media containing 17 µg/µl Cam at 42 °C over night. Over night cultures were diluted 1:1000 in liquid LB without antibiotics, grown to an OD_{600} of 0.5 and then plated out on LB plates. Colonies from these plates were replica-plated on 17 µg/µl Cam plates and LB plates and chromosomal DNA from Cam^s colonies were tested by PCR employing primers 7 and 8 (flanking the *fimE* gene). A colony, which had the normal *fimE* gene was selected and named HEHA6 (Fig. 1C).

Colony morphology. The colony morphology was assayed by employing a Carl Zeiss Axioplan epifluorescence microscope and digital images were captured with a 12-bit cooled slow-scan

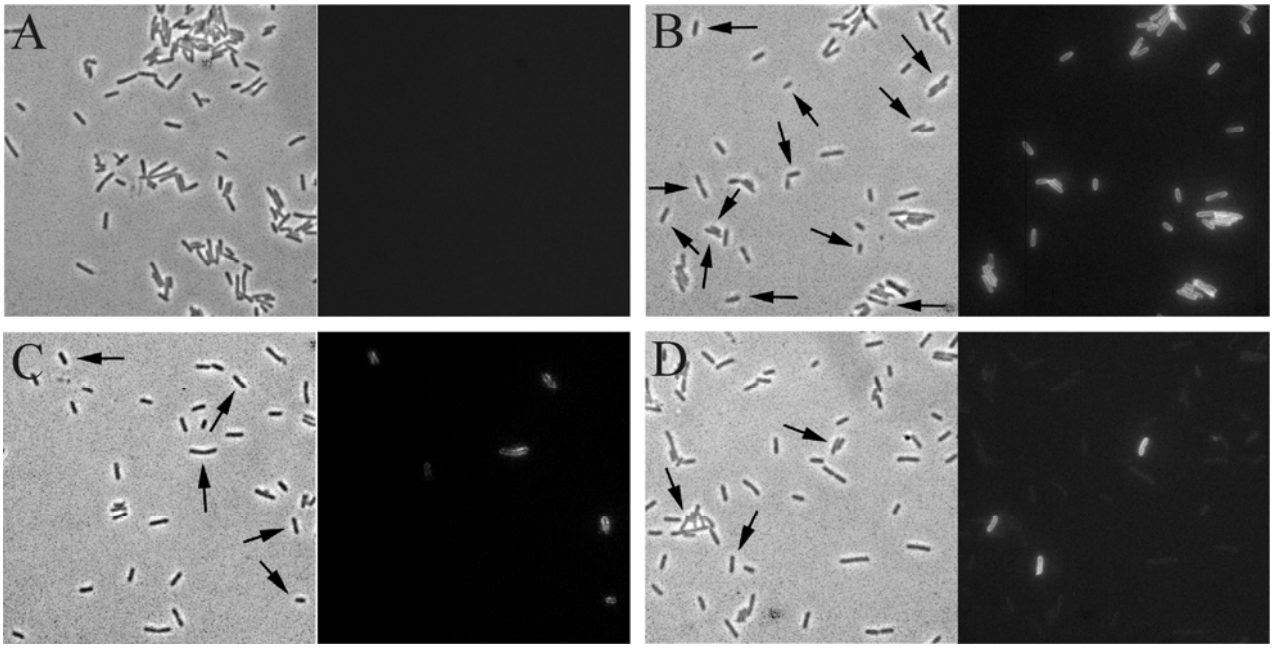

Fig. 7. Phase contrast microscopy (left part of figures) and immunofluorescence microscopy (right part of figures) of overnight cultures of strain BD1512 (A) and (B) and strain BD1511 (C) and (D) employing primary antibodies directed against either FimA (A) and (C) or Ag43 (B) and (D) and FITC-labeled secondary antibodies. Black arrows indicate individual cells, which exhibit a different phenotype than the majority.

charge-coupled device (CCD) camera (KAF 1400 chip; Photometrics, Tucson, Arizona) controlled by the PMIS software (Photometrics).

Immunofluorescence microscopy. Surface presentation of type 1 fimbriae and Ag43, respectively, was assessed by immunofluorescence microscopy employing a monoclonal antibody directed against FimA (Stentebjerg-Olesen *et al*., 1997) or a polyclonal serum that recognizes the α-subunit of Ag43 (a kind gift from Peter Owen). Cell fixation, immunolabeling and microscopy was carried out as previously described (Pallesen *et al*., 1995) employing a FITC-labeled secondary antibody.

RESULTS

Expression of type 1 fimbriae and Ag43 in strain BD1511/BD1512. The *E. coli* strains BD1511 and BD1512 are phase variants of the same strain; as such they are genetically identical but phenotypically distinct. They differ by the following criteria: BD1511 forms small glossy colonies (Fig. 1) and promotes yeast agglutination indicative of type 1 fimbriation whereas BD1512 forms large frizzy colonies (Fig. 1) and does not agglutinate yeast cells. Furthermore, only the BD1512 variant auto-aggregates in liquid suspension. Overnight cultures of a glossy (Form 2) and a frizzy (Form 1) colony, respectively, were prepared (c.f. Material and Methods) and tested for expression of type 1 fimbriae and Ag43, respectively, by immunofluorescence microscopy (Fig. 3). As can be seen, cells originating from a Form 1 colony (BD1512) do not express type 1 fimbriae (Fig. 3A) and more than 3/4 of the cells expressed Ag43 (Fig. 3B). Of the cells originating from a Form 2 colony (BD1511), only a small percentage expressed either type 1 fimbriae (Fig. 3C) or Ag43 on the surface (Fig 3D). Unfortunately the assay required the cells to grow for at least 16 hours in liquid media, which allow cells to undergo phase variation and convert to a different phenotype from the one predominant in the colony (see later). To circumvent this problem, colonies were picked directly from the plates (Fig. 4A and 4D), prepared for immuno-fluorescence microscopy and tested for expression of type 1 fimbriae and Ag43. This revealed, that most (>95%) of the cells from a Form 1 colony expressed Ag43 but not type 1 fimbriae (Fig. 4B and 4C), whereas cells from a Form 2 colony showed the opposite relationship, *viz*. they were virtually all fimbriated (>99%) but only a few (<5%) expressed Ag43 (Fig. 3E and 3F). These results indicate that Form 1 cells from large frizzy colonies are Fim^-, $Ag43^+$ and Form 2 cells from small glossy colonies are Fim^+, $Ag43^-$.

The phase variable morphologies of BD1511/BD1512. The phase variable colony morphology of the BD1511/BD1512 strain set was monitored by CCD microscopy (Fig. 5). The BD1511 variant gives rise to small, high, circular and glossy colonies (Fig. 5A) and a larger variant that was flat and had more irregular edges (Form

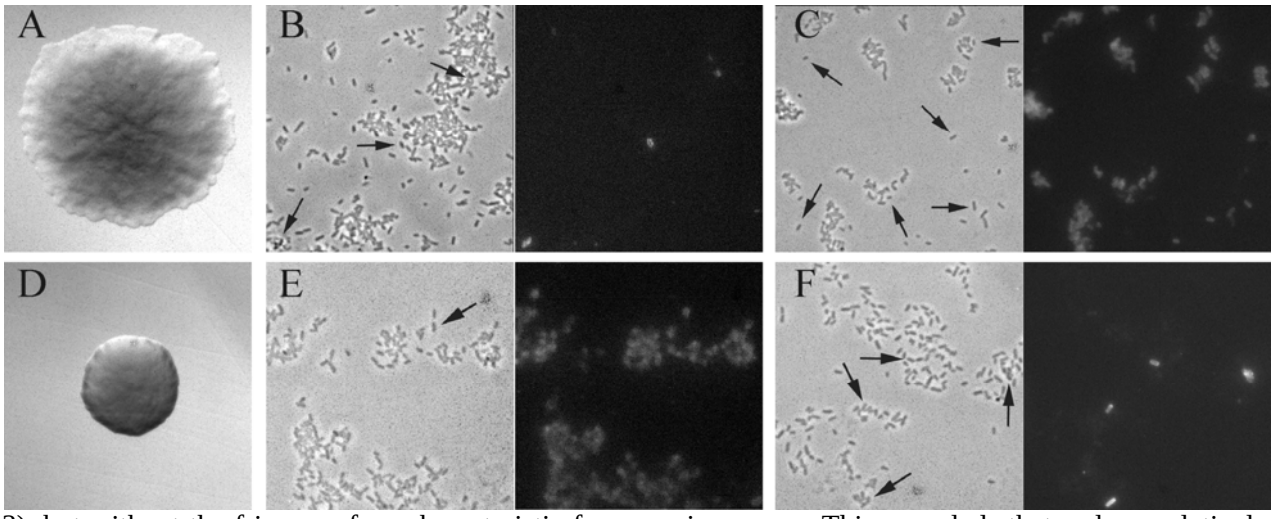

Fig. 8. Phase contrast microscopy (left part of figures) and immunofluorescence microscopy (right part of figures) of selected colonies of strain BD1512 (B) and (C) and strain BD1511 (E) and (F) employing primary antibodies directed against either FimA (B) and (E) or Ag43 (C) and (F) and FITC-labeled secondary antibodies. Black arrows indicate individual cells, which exhibit a different phenotype than the majority. CCD images of the selected colonies of BD1512 (A) and BD1511 (D) are shown to the left.

3), but without the frizzy surface characteristic for Form 1 (Fig. 5B). BD1512 also showed phase variation of the colonies (Fig. 5C and 5D), but here both types were flat and equal in size and could only be distinguished by their surface appearances, as some of them had extremely frizzy surfaces and some had a smooth surface (Fig. 5D). Interestingly, neither of the two colony forms (Form 1 and Form 2) was able to convert directly to the other upon a single re-plating. Rather, the shift from Form 1 to Form 2 and vice versa, occurred through a transition state (Form 3; Fig. 5A and Fig. 5D) which was characterized by a colony morphology with a flat and irregular shape but with a smooth surface. The complete transition from Form 1 to Form 2, or from Form 2 to Form 1, only took place after repeated re-streaking (at least 3 times) of a Form 3 colony type.

Ag43 phenotype of Form 3 colonies. In order to test the ability to produce Ag43, a simple auto-aggregation assay was performed with shaken overnight cultures (c.f. Material and Methods) of the three different colony types from BD1511 and BD1512 (Form 1, Form 2 and Form 3). The progeny of a Form 1 colony could be either Form 1 or Form 3 (Fig. 6). The auto-aggregation assay showed that only the Form 1 cells were able to auto-aggregate. The progeny of a Form 2 colony could be either Form 2 or Form 3 (Fig. 6). The progeny originating from a Form 2 colony was unable to auto-aggregate, even if the resulting colony had shifted to Form 3. This suggested that Form 3 did not produce Ag43. To address this, Form 3 colonies originating from both a Form 1 and a Form 2 colony were selected (Fig. 7A and 7D) and submitted to immuno-fluorescence microscopy. This revealed, that only a relatively small fraction of the Form 3 cells expressed Ag43 on the surface (Fig. 7C and 7F), with a tendency of colonies originating from Form 1 (Fig. 7C) to contain more cells expressing Ag43 (5-10%) than the ones originating from Form 2 (<2%; Fig. 7F). Furthermore only a very small fraction (<2%) of Form 3 cells expressed type 1 fimbriae (Fig. 7B and 7E) indifferent of whether they originated from a Form 1 or Form 2 colony originally

A *flu*-null mutant is incapable of producing frizzy colonies. In order to investigate the role of the *flu* locus in colony morphology, a mutant of the BD1511/BD1512 strain set, HEHA11, was made, in which the *flu* gene was knocked out by insertion of a *tet* cassette (c.f. Material and Methods). Interestingly HEHA11 exhibited both Form 2 and Form 3 colony morphology (Fig. 5E and 5F), however the ability to produce Form 1 colonies appeared to be lost, even after consecutive re-streaking of Form 3 colonies. This result was further corroborated by reintroducing the *flu* gene on a plasmid (pHHA146) in HEHA11, which reinstalled the ability to form the frizzy phenotype (Fig. 5G and 5H). This clearly indicates that the *flu* gene was required in order to produce a frizzy phenotype, however, the *flu* gene seemed to play no role in the colony-size phenotype.

OxyR-influence on colony morphology. Strain BD1302 has a deletion encompassing the *oxyR* gene, which cause constitutive expression of Ag43, thus locking the colonies of BD1302 in Form 1. Whether OxyR influenced Ag43 expression and colony morphology in the BD1511/BD1512 strain set was investigated by constructing a defined *oxyR* mutant, HEHA9, in which the *oxyR* gene was

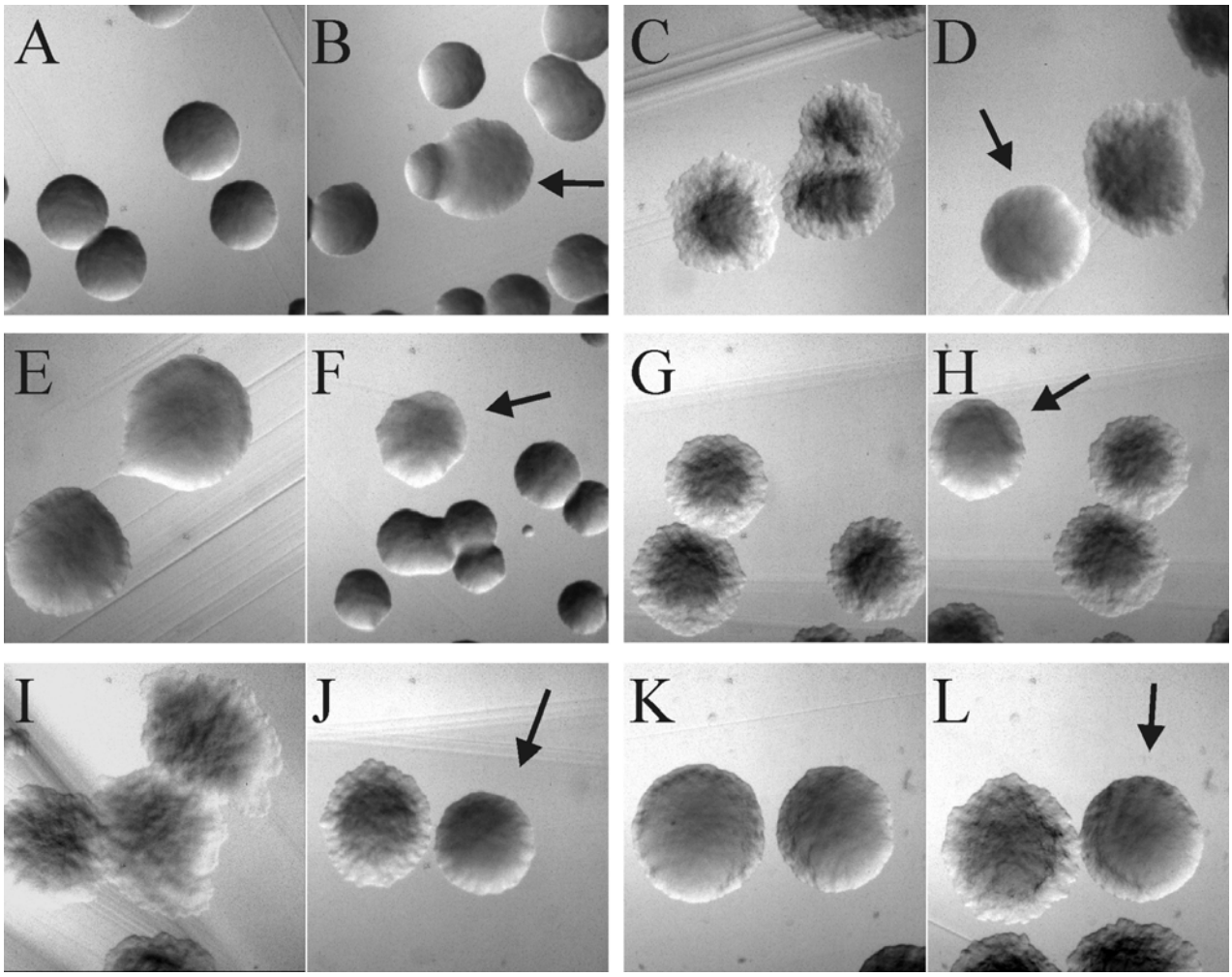

Fig. 5. CCD images of selected colonies of various strains: BD1511 (A) and (B), BD1512 (C) and (D), HEHA11 (E) and (F), HEHA11(pHHA146) (G) and (H), HEHA9 (I), HEHA4 (J), HEHA6 (K) and (L). See text for details. Black arrows indicates colonies, which are different from the majority.

replaced by an *oxyR::spectinomycin* cassette (c.f. Material and Methods). Strain HEHA9 exclusively gave rise to Form 1 colonies (Fig. 5I) on LB plates and all of these were constitutive Ag43-expressors leading to auto-aggregating in liquid suspension, thus mimicking the observed phenotype of BD1302.

Influence of fimbriae expression on colony morphology. The data presented above indicated that only Form 2 colonies expressed type 1 fimbriae, suggesting a role for type 1 fimbriation in colony size. To examine this tenet further, a *fim* deletion mutant, HEHA4, of the strain set BD1511/BD1512 was made (c.f. Material and Methods). After several rounds of restreaking of this strain, it became clear, that HEHA4 was still subject to phase variation between Form 1 and Form 3, but Form 2 colonies were not seen (Fig. 5J). This further supported the involvement of type 1 fimbriae in generating Form 2 colonies. When Form 1 and Form 3 colonies were grown in liquid media overnight, only the cells from Form 1 colonies auto-aggregated as expected if the Ag43-mediated auto-aggregation was unaltered by deleting the *fim* gene.

The *fimE* gene of strain BD1511/BD1512 is truncated by an IS*1* element. During the construction of the *fim* mutant, HEHA4, it became clear, that the BD1511/BD1512 strain set carried an insert of about 750 bp in the start of the *fimE* gene. Subsequent sequencing of this region revealed the presence of an IS*1* element after the 12^{th} base in the *fimE* gene, thus rendering this gene non-functional. This in fact mimicked the situation in strain CSH50, which also has an IS*1* insertion in the *fimE* gene, although in a different position. By allelic exchange, the truncated *fimE* gene in BD1511 was replaced with the wild type *fimE* gene from *E. coli* K-12 strain PC31 resulting in strain HEHA6, thereby restoring the integrity of the *fim* gene cluster (c.f. Material and Methods). Colonies of strain HEHA6 exhibited phase variation between Form 1 and Form 3, but notably Form 2 was not present (Fig. 5K and 5L). Again, only

Form 1 colonies gave rise to auto-aggregation in liquid suspensions. This indicates that the presence of FimB in the absence of FimE accounts for the ability to vary between small and large colony sizes.

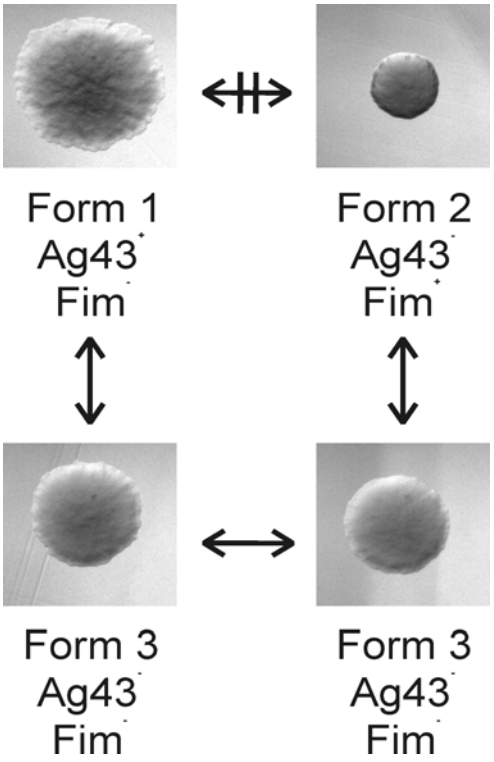

Fig. 6. Schematic representation of the three different colony morphologies of BD1511/ BD1512 and their Ag43 status. (A) The progeny of a Form 1 colony (BD1512) is either Form 1 or Form 3. Only cells in a Form 1 colony express AG43. (B) The progeny of a Form 2 colony (BD1511) is either Form 2 or Form 3. Neither of these express Ag43.

DISCUSSION

Remarkably little attention has been given to colony morphology variation of *E. coli* (and other bacteria) and the underlying mechanisms. Two factors reported to influence colony morphology of certain *E. coli* K-12 strains, are in fact, both phase variable surface components, *viz.* antigen 43 and type 1 fimbriae. Despite their different expression control mechanisms, previous reports have indicated a correlation between these two surface structures and the ability of certain *E. coli* K-12 strains to produce two distinct colony morphologies (Brinton, 1965; Diderichsen, 1980; Orndorff and Falkow, 1984; Blomfield *et al*., 1991). One is small, high, has a circular edge and smooth surface (called *glossy* or Form 2), the other is large, flat, has a jagged edge and rough surface (called *frizzy* or Form 1) and these two colony morphologies are interchanging. Examination of cells from glossy colonies reveals, that they agglutinate yeast cells (indicative of type 1 fimbriation) and do not form auto-aggregates in liquid suspensions (indicative of a $Ag43^-$ phenotype), whereas cells from frizzy colonies does not agglutinate yeast cells (Fim^-) but auto-aggregate ($Ag43^+$).

The roles of the individual components (Ag43 and type 1 fimbriae) were investigated by constructing deletion mutants and examine the resulting colony morphologies. Additionally, selected clones were subjected to immunofluorescence microscopy employing specific antibodies directed against either Ag43 or type 1 fimbriae.

From the beginning it became clear, that it was necessary to pick cells directly from the colonies, and not from overnight cultures, if the precise type

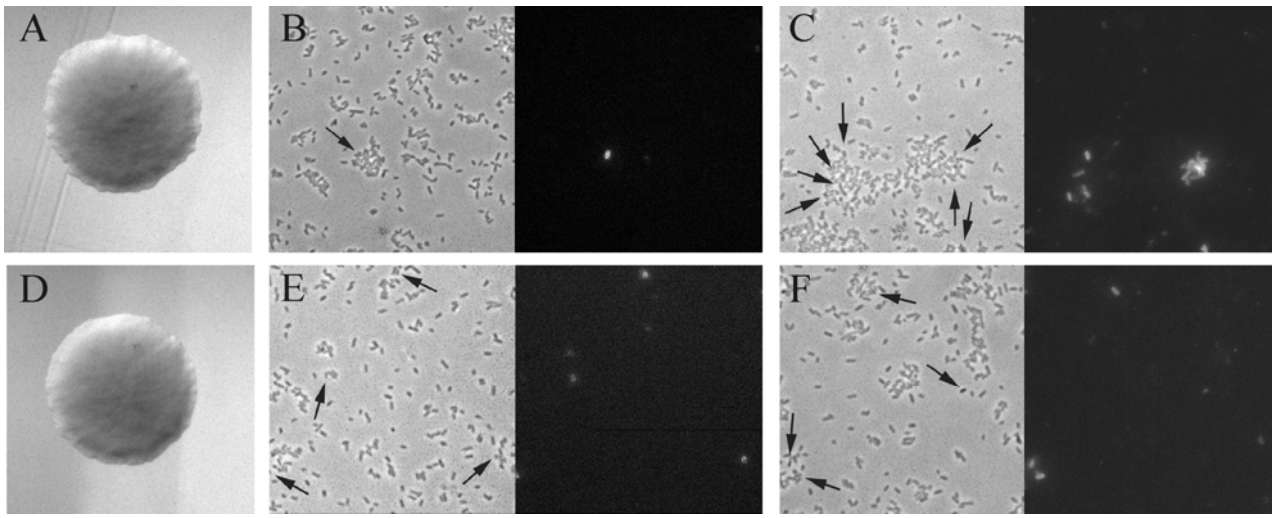

Fig. 7. Phase contrast microscopy (left part of figures) and immunofluorescence microscopy (right part of figures) of selected Form 3 colonies originating from a Form 1 colony (BD1512) (B) and (C) and a Form 2 colony (BD1511) (E) and (F) employing primary antibodies directed against either FimA (B) and (E) or Ag43 (C) and (F) and FITC-labeled secondary antibodies. Black arrows indicate individual cells, which exhibit a different phenotype than the majority. CCD images of the selected Form 3 colonies originating from either BD1512 (A) or BD1511 (D) are shown to the left.

1 fimbriation and Ag43 status was to be examined. When this was done, a somewhat different picture (than seen with overnight cultures) emerged, notably in the case of cells from a Form 2 colony. Here, most of the cells expressed type 1 fimbriae in contrast to the liquid overnight cultures. This suggests that type 1 fimbriae expression is more sensible to the environmental changes caused by the liquid growth than Ag43, thus underlining the differences in their regulation.

While examining the Form 1 and Form 2 colonies it became evident, that they interchanged through a transition state (Form 3), which was a hybrid between the two original forms. Form 3 was characterized by large, flat colonies with a jagged edge and a smooth surface and the cells were unable to auto-aggregate. Immunofluorescence microscopy of a Form 3 colony originating from both a Form 1 and a Form 2 colony showed, that few of the cells expressed either type 1 fimbriae or Ag43. More cells from a Form 3 colony, which originated from a Form 1 colony produced Ag43 on the surface than cells from a Form 3 colony originating from a Form 2 colony. In both cases, the Ag43 expressing cells totaled less than 5% showing that the Ag43 production in Form 3 colonies is more or less turned of and that this seemed to happen gradually (*viz.* Form 1→ Form 3 (from Form 1) → Form 3 (from Form 2) → Form 2).

OxyR has been shown to repress Ag43 expression. A strain, BD1302, carrying a large deletion the chromosomal region containing the *oxyR* gene was shown to be a constitutive expressor of Ag43 and to be locked in the Form 1 state. The present data indicate that this is solely due to the *oxyR* mutation. This became clear because a defined *oxyR* mutant derivative of the BD1511/BD1512 strain set was observed to be locked in the Form 1 state. Furthermore, reintroducing the *oxyR* gene on a plasmid restored the phase varying phenotype seen in the wild type strain. However, these experiments do not rule out, that the effect could be indirect as a result of OxyR regulating another protein (such as for example the small regulatory RNA called *oxyS*), which might then regulate the *flu* gene. The similarity of the *flu* promoter with the *mom* promoter of phage Mu (Henderson and Owen, 1999), which has been shown to bind OxyR (Bölker and Kahmann, 1989), however, strongly indicate a direct regulation mechanism of OxyR on the *flu* gene.

To further investigate whether Ag43 or type 1 fimbriae expression influenced the shift between colony morphologies, deletion mutants of both of the respective loci were made and the resulting colonies were examined by light microscopy. A *tet* antibiotic resistance cassette was inserted into the *flu* gene of the strain set BD1511/BD1512 (resulting in the strain HEHA11). Cells of this strain were seen to vary between a Form 2 and a Form 3 colony type, but colonies exhibiting Form 1 were not seen. Only when the *flu* gene was reintroduced into HEHA11 could Form 1 be seen. This showed that Ag43 was required for the frizzy phenotype of the colonies, but was not involved in size variation. Deletion of the *fim* gene cluster in the BD1511/BD1512 strain set (strain HEHA4) resulted in cells, which were able to shift between a Form 1 and a Form 3 morphology, this time excluding Form 2 colonies. This fit well with the fact that only cells from Form 2 colonies (of BD1511) express type 1 fimbriae on their surface. A possible explanation for this phenomenon was found, when the chromosomally located *fim* gene cluster of the BD1511/BD1512 strain set was examined. It turned out, that an IS*1* insertion element was located within the *fimE* gene, encoding for one of the recombinases, that are partially responsible for the phase variation of type 1 fimbriae. This indicates that it is only the other recombinase (FimB), which is functional in this strain. A similar situation has been reported in another *E. coli* strain (CSH50), which also has a truncated *fimE* gene due to IS*1* element insertion (Blomfield *et al*., 1991). CSH50 is likewise subject to colony phase variation, and transferring the truncated *fimE* into a strain (MG1655), where the *fim* gene cluster is intact and which has uniformly looking colonies, has been shown to lead to colony phase variation in this strain as well (Blomfield *et al*., 1991). In this study the opposite approach was tried since a functional *fimE* gene was introduced into the chromosome of the BD1511/BD1512 strain set by allelic exchange thereby creating the strain HEHA6. This led to colony morphologies similar to what was seen when introducing the complete *fim* gene cluster into the *fim* deletion mutant. Therefore, Form 2 colonies only appear in *fimE* mutants. It has previously been shown that the biochemical activity of the FimE recombinase is significantly higher than that of FimB. Thus on solid media FimE conferred ON-to-OFF phase switching by a frequency as high as 0.75/cell/generation. For comparison FimB-mediated ON-to-OFF and OFF-to-ON switching was reported to be around 10^{-3}/cell/generation (Gally *et al*., 1993). This phenomenon might explain why the Form 2 colony morphology associated with type 1 fimbriation is only observed in *fimE* mutants. When FimE is present it cause fast ON-to-OFF switching essentially resulting in non-fimbriated cells and thus abolishing Form 2 colonies.

A recent study on the behavior of *Pseudomonas fluorescence* cells belonging to different morph

types identified direct correlation between colony morphology and life style in static liquid media (Rainy and Travisano, 1998). It is therefore interesting to correlate the colony phenotype reported in this study, with life styles in static liquid environments. Static liquid conditions are encountered by *E. coli* for example when mammals defecate in stagnate water bodies i.e. pools, ponds lakes etc. From this perspective Form 1 cells, characterized by a $Ag43^{+}$, Fim^{-} phenotype, would represent a bottom dwelling community, since Ag43 confers auto-aggregation and settling of the bacteria (Hasman *et al*., Chapter 2). On the contrary, Form 2 cells, characterized by a $Ag43^{-}$, Fim^{+} phenotype, would be surface dwellers due to the induction of surface-pellicle by type 1 fimbriated cells (Old and Duguid, 1970; Harris *et al*., 1990). Finally, Form 3 cells, being $Ag43^{-}$ and Fim^{-}, would represent a "pelagic" sub-population, with no particular preference for surface or bottom life style.

In conclusion both Ag43 and type 1 fimbriation is involved in the colony morphology formation. Ag43 is responsible for the rough surface appearance of Form 1 colonies and type 1 fimbriation is responsible for Form 2 colony formation.

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Concluding remarks

Concluding remarks

In this thesis I have followed two partly overlapping scientific aims, which have been summoned into three separate chapters. The first chapter describes the means by which I have improved the capacity of *E. coli*, and its type 1 fimbriae, to accommodate more FimH than is normally possible. The second chapter deals with Antigen 43 (Ag43) and the interaction of this member of the autotransporter family with type 1 fimbriae. Finally, chapter three describes the origin of the phase variation, which leads to the differences in colony morphology observed amongst certain *E. coli* strains.

Overexpression of the potential vaccine component, the FimH adhesin, on the surface of *Escherichia coli* by redesign of the *fim* gene cluster.

It has proven extremely difficult to increase the FimH content in *E. coli*. This is due to the fact, that FimH depends upon other fimbrial components in order to be incorporated into the fimbrial shaft. Expression of FimH from the normal *fim* gene cluster is first of all restricted by the *fimH* gene's position as the most distal gene, with regards to the *fim* promoter. I made transcriptional fusions of the reporter gene, *lacZ*, to several different positions within the *fim* gene cluster and showed that there was a 50-fold difference between transcription of the *fimA* gene and the *fimH* gene. Therefore, the *fimH* gene was moved to two different positions closer to the *fimA* gene: one just in front of *fimA* and one immediately behind it. Northern blot analysis revealed that only the latter position led to transcription of the *fimH* gene at comparable levels to that of the *fimA* transcript. This, however, did not lead to an increase in the production of FimH. It was concluded that this was a result of the other fimbrial components not being present and therefore a bottleneck occurred in the periplasm and proteolytic proteins rapidly degraded excess FimH. To avoid this, the amount of fimbrial periplasmic chaperones and outer membrane proteins, FimC and FimD, respectively, were also increased. This increased the FimH content of the cells significantly, but it did not increase the content of FimH within the fimbriae. To achieve this, it was also necessary to increase the amount of the two minor components, FimF and FimG, together with FimC and FimD. This eventually led to a 25-fold increase in FimH content within total cell lysates and a 10-fold increase in FimH within the fimbriae. To illustrate that this could also be achieved in a possible vaccine candidate strain, the experiments were simultaneously carried out in *E. coli* Nissle 1917, a strain which has been used extensively as a probiotic in Germany for decades.

The potential outcome of this work could be the construction of vaccine candidate strains with an increased FimH content. Such strains may elicit an immune response against the FimH protein, which could provide protection against pathogenic *E. coli*, as previously shown with truncated FimH proteins (Langermann *et al*., 1997; Thankavel *et al*., 1997). This could be accomplished either by employing non-pathogenic strains, such as the *E. coli* Nissle 1917, or by purifying the fimbriae from overexpressing strains and then administrating these proteins as vaccine components. Another possibility is to use the described constructs as carriers of foreign epitopes, such as the cholera toxin B subunit, in a manner similar to that described for FimA (Stentebjerg-Olesen *et al*., 1997). Finally, such constructs could be used to insert metal binding domains as has been demonstrated recently (Schembri *et al*., 1999), thereby increasing the metal binding capacity of the bacteria.

Antigen 43 Mediated Auto-Aggregation of *Escherichia coli* is Neutralized by Type 1 Fimbriation.

Antigen 43 belongs to a small group of protein complexes called the autotransporters. It consists of two separate subunits, denoted the α and the β subunit, which are transcribed together as a preprotein from the *flu* gene. The β subunit is located in the outer membrane and is believed to act as a pore for the α subunit (Henderson and Owen, 1999). Expression of Ag43 leads to auto-aggregation of the bacteria (Diderichsen, 1980). In chapter two I have shown, by an auto-aggregation assay and the use of immunofluorescence microscopy, that this auto-aggregation is solely due to the *flu* gene product (Ag43), and that this is the only component required to obtain this phenomenon.

Ag43 is, like type 1 fimbriation, subject to phase variation, but the regulatory mechanism is different. Introduction of one of the regulatory genes, FimB, from the *fim* gene cluster does, nevertheless, abolish the ability of Ag43-expressing cells to auto-aggregate. This was shown by immunofluorescence microscopy, to be as a result of fimbriae on the cell surface, and not as part of a common regulatory mechanism involving repression of Ag43 by FimB.

A small part of this chapter has, after it was submitted to the Journal of Bacteriology, been published by others (Henderson and Owen, 1999), even though it describes a slightly different *flu* allele. Of course this was not known when the article was submitted and does not detract from the results of the present paper. The overlap is mainly regarding the size of the *flu* gene, the sizes of the α and β subunits and the identification of the presumed promoter. With regards to the sizes, the two articles are in fine agreement (except from an error in subunit sizes of Henderson and Owens discussion!). The promoter predicted by the two papers, however, is not the same. One of the papers (Henderson and Owen, 1999), predicts a promoter with good –35 and –10 sequences, but they are only separated by 14 bp, which is highly unlikely to be enough to ensure a strong recognition of the RNA polymerase (Auble and deHaseth, 1986; Dombroski *et al*., 1996). I (Chapter two) propose alternative –35 and –10 sequences, and these are separated by a more optimal 17 bp spacer. This promoter sequence is furthermore conserved within both *flu* alleles, which is not the case with the promoter proposed by Henderson and Owen. To conclusively examine this matter of difference, primer extensions will have to be performed.

The Role of Antigen 43 and Type 1 Fimbriation in *Escherichia coli* Colony Morphology.

Both Ag43 and type 1 fimbriae have been suggested to determine the colony morphology formation of *E. coli* (Brinton, 1965; Diderichsen, 1980). Some *E. coli* strains phase vary between two different colony morphologies. One is large, rough, flat, irregular and generates auto-aggregation of liquid suspensions (called *frizzy* or Form 1) and the other is small, smooth, raised, circular and is non-aggregating (called *glossy* or Form 2). I discovered that a third form (called Form 3) also exists. This was characterized by large, smooth, flat and circular colonies. When all three forms were examined by immunofluorescence microscopy, it became clear that cells from a Form 1 colony were $Ag43^{+}$, Fim^{-}, cells from a Form 2 colony were $Ag43^{-}$, Fim^{+} and cells from a Form 3 colony were $Ag43^{-}$, Fim^{-}.

To assess the individual roles of Ag43 and type 1 fimbriae, deletion mutants of both were constructed and the resulting colonies were examined. This revealed that Ag43 was not responsible for the variation of the colony sizes, but that it gave the colonies their rough surface. The phase variation of the colony sizes was shown to be connected to type 1 fimbriation of the cells, as the

Δ*fim* strain proved unable to form small (Form 2) colonies. Further examination of the *fim* gene cluster of the phase varying strain set (BD1511/BD1512) revealed tat the *fimE* gene harbored an IS*1* element, thus abolishing FimE expression. When the mutated *fimE* gene was replaced by a functional allele from the *E. coli* strain PC31, the resulting strain was again unable to produce Form 2 colonies. This is most likely as a result of the high switching frequency towards the OFF orientation promoted by FimE as compared to FimB (Gally *et al*., 1993). This also explains why most *E. coli* strains are not observed to exhibit different colony sizes, as they all carry an intact *fim* gene cluster.

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