Cellular chain formation in \textit{Escherichia coli} biofilms

Rebecca Munk Vejborg and Per Klemm

Microbial Genomics Group, Centre for Biological Sequence Analysis, Department of Systems Biology, Technical University of Denmark, Building 301, DK-2800 Kgs. Lyngby, Denmark

INTRODUCTION

Many bacteria live in highly complex sessile communities, referred to as biofilms (Costerton et al., 1978; Geesey et al., 1977). These compact microbial consortia often confer certain advantages on the microbial population, such as antibiotic resistance, immune evasion, shear resistance and general persistence in changing and often hostile environments (Costerton et al., 1995, 1999). As such, microbial biofilms constitute a significant problem in various industrial and food-related areas, where microbial colonization often imposes a considerable financial burden (Donlan & Costerton, 2002; Lee Wong, 1998). Microbial biofilm formation has also been implicated in the development and persistence of a range of both implant- and non-implant-related infections (Costerton et al., 1999). This has warranted a greater understanding of the importance of microbial biofilms in both health and disease, and of biofilm formation in itself.

\textit{Escherichia coli} is a genetically diverse microbial species. While \textit{E. coli} is usually associated with a commensal lifestyle in the gastrointestinal tract, some \textit{E. coli} strains have acquired specific virulence attributes that enable them to adapt to and colonize distinct host niches. Pathogenic \textit{E. coli} causes a range of intestinal infectious diseases, usually manifested by diarrhoeal and dysentery-like symptoms (Kaper et al., 2004). \textit{E. coli} is also responsible for a range of extra-intestinal infections, including urinary tract infections (UTIs), septicemia and neonatal meningitis (Kaper et al., 2004). Several studies have suggested that biofilm formation is important for the development or persistence of some \textit{E. coli}-associated infectious diseases. Intracellular biofilms, for instance, may be important for the development of symptomatic UTIs (Anderson et al., 2003).

Bacterial biofilm formation is a complex process involving a multitude of molecular factors and physiological events. It generally proceeds through a series of developmental stages including bacterial adhesion, proliferation and structural maturation (O’Toole et al., 2000). Recent studies have suggested that the spatiotemporal organization of the biofilm and the formation of distinct bacterial subpopulations are important for the biofilm developmental process, and for the phenotypic characteristics of a mature biofilm (Haagensen et al., 2007; Pamp et al., 2008). The final biofilm architecture displayed by various bacterial species and strains, however, varies extensively, suggesting that biofilm development is a largely strain- and environment-specific process (O’Toole & Kolter, 1998; Reisner et al., 2006).

Among \textit{E. coli} strains, a range of biofilm structural phenotypes can also be observed, from simple flat, compact

Abbreviations: ABU, asymptomatic bacteriuria; Ag43, antigen 43; FLIP, fluorescence loss in photo-bleaching; UPEC, uropathogenic \textit{E. coli}; UTI, urinary tract infection.

Details of minimal media and primers are available as supplementary material with the online version of this paper.
biofilms to elaborate, loose biofilm structures encompassing long protruding structures (Reisner et al., 2006). Several surface organelles, including various types of fimbriae, autotransporter proteins and extracellular polysaccharides, have been found to facilitate or enhance biofilm formation of *E. coli*, largely depending on the environmental conditions and the particular strains studied (Schembri et al., 2002). Many more uncharacterized adhesins are predicted to exist. During biofilm studies in our laboratory, we have observed that an *E. coli* K-12 strain, SAR19, can display extensive cellular chaining during biofilm formation. This has not previously been observed in *E. coli*, and the purpose of this study was therefore to characterize this unique biofilm phenotype further. Although cellular chain formation was studied mainly in *E. coli* K-12 strains, the phenomenon does not appear to be restricted to K-12, illustrating the diversity in biofilm architectures observed even within a single bacterial species.

**METHODS**

**Bacterial strains and growth conditions.** The strains employed in the study are listed in Table 1. Modified Luria–Bertani (LB) broth, minimal media (FAB, M63, M9 or MOPS; see supplementary data, available with the online version of this paper, for details) (Kjaergaard et al., 2000b; Miller, 1992; Neidhardt et al., 1974), containing 0.2 % glucose, thiamine (1 μg ml⁻¹) and proline (10 μg ml⁻¹), or pooled sterile filtered urine were used for overnight cultivation. Overnight cultures were always established under the same experimental conditions [temperature (30 °C or 37 °C), medium (but always with 0.2 % glucose)] as were used in the subsequent biofilm experiments. Antibiotics were added when required in the following concentra-

### Table 1. Strains and plasmid used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>K-12 strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSH26sm</td>
<td>Spontaneous SmR mutant of CSH26</td>
<td>Reisner et al. (2003)</td>
</tr>
<tr>
<td>SAR19</td>
<td>attB:: bla-rrnBP1-cfp-Tₜ₀; AmpR</td>
<td>Reisner et al. (2003)</td>
</tr>
<tr>
<td>SAR20</td>
<td>attB:: bla-rrnBP1-yfp-Tₜ₀; AmpR</td>
<td>Reisner et al. (2003)</td>
</tr>
<tr>
<td>CSH26Δflu</td>
<td>CSH26Δflu</td>
<td>This study</td>
</tr>
<tr>
<td>CSH26Δflu, cfp</td>
<td>CSH26Δflu, attB:: bla-rrnBP1-cfp-Tₜ₀; AmpR</td>
<td>This study</td>
</tr>
<tr>
<td>CSH26csgAB</td>
<td>CSH26csgAB:: kanR</td>
<td>This study</td>
</tr>
<tr>
<td>CSH26fim</td>
<td>CSH26fim:: kanR</td>
<td>This study</td>
</tr>
<tr>
<td>CSH26cps</td>
<td>CSH26waeDE:: kanR</td>
<td>This study</td>
</tr>
<tr>
<td>MS427</td>
<td>MG1655Δflu</td>
<td>Reisner et al. (2003)</td>
</tr>
<tr>
<td>MC4100</td>
<td>araD139 Δ(argF-lac)U169 rpsL150 relAdeoC1 rbsR22 fruA25</td>
<td>Lab. strain collection</td>
</tr>
<tr>
<td>ZK2686</td>
<td>W3110 Δ(argF-lac)U169</td>
<td>Danese et al. (2000b)</td>
</tr>
<tr>
<td><strong>UTI isolates</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFT073</td>
<td>O6:: K2:: H1</td>
<td>Welch et al. (2002)</td>
</tr>
<tr>
<td>NU14</td>
<td>O18:: K1:: H7</td>
<td>Hulgren et al. (1986)</td>
</tr>
<tr>
<td>83972</td>
<td>OR:: K3:: H⁻</td>
<td>Lindberg et al. (1975)</td>
</tr>
<tr>
<td>83972yfp</td>
<td>83972 attB:: bla-rrnBP1-yfp-Tₜ₀; AmpR</td>
<td>Hancock et al. (2007)</td>
</tr>
<tr>
<td>1177</td>
<td>O1:: K1:: H7</td>
<td>Marild et al. (1989)</td>
</tr>
<tr>
<td>J96</td>
<td>O4:: K6</td>
<td>Hull et al. (1981)</td>
</tr>
<tr>
<td>VR50</td>
<td>OR:: K1:: H⁻</td>
<td>Roos et al. (2006)</td>
</tr>
<tr>
<td>VR89</td>
<td>OR:: H⁻</td>
<td>Roos et al. (2006)</td>
</tr>
<tr>
<td>VR90</td>
<td>O14:: H⁻</td>
<td>Roos et al. (2006)</td>
</tr>
<tr>
<td>VR91</td>
<td>O134:: H16</td>
<td>Roos et al. (2006)</td>
</tr>
<tr>
<td>VR92</td>
<td>OR:: H⁻</td>
<td>Roos et al. (2006)</td>
</tr>
<tr>
<td>VR94</td>
<td>O18ac:: K1:: H⁻</td>
<td>Roos et al. (2006)</td>
</tr>
<tr>
<td>VR95</td>
<td>O25:: H2</td>
<td>Roos et al. (2006)</td>
</tr>
<tr>
<td>VR96</td>
<td>O134:: H16</td>
<td>Roos et al. (2006)</td>
</tr>
<tr>
<td>VR136</td>
<td>ABU isolate</td>
<td>Lab. strain collection</td>
</tr>
<tr>
<td>VR137</td>
<td>ABU isolate</td>
<td>Lab. strain collection</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBR322</td>
<td>Cloning vector, AmpR</td>
<td>Bolivar et al. (1977)</td>
</tr>
<tr>
<td>pACYC184</td>
<td>Cloning vector, CamR</td>
<td>Chang &amp; Cohen (1978)</td>
</tr>
<tr>
<td>pBADmyc-HisA</td>
<td>Commercial cloning vector, AmpR</td>
<td>Guzman et al. (1995)</td>
</tr>
<tr>
<td>pPKL4</td>
<td>fim operon in pBR322</td>
<td>Klemm et al. (1985)</td>
</tr>
<tr>
<td>pKKJ128</td>
<td>flu gene in pACYC184</td>
<td>Kjaergaard et al. (2000a)</td>
</tr>
<tr>
<td>pKKJ143</td>
<td>flu gene in pBAD/myc-HisA</td>
<td>Kjaergaard et al. (2002)</td>
</tr>
</tbody>
</table>
tions: kanamycin (25 μg ml⁻¹), chloramphenicol (20 or 25 μg ml⁻¹), ampicillin (100 μg ml⁻¹). Static biofilms were cultured in LB or FAB minimal medium containing 0.2% glucose, thiamine (1 μg ml⁻¹) and proline (10 μg ml⁻¹).

Recombinant DNA techniques. The CSH26 knockout mutants were constructed using the λ-red recombinease-based gene inactivation system (see supplementary data) (Datsenko & Wanner, 2000). The knockout mutations were confirmed by PCR. *E. coli* CSH26Δflu was fluorescently tagged by insertion of a cfp-containing cassette in the bacteriophage λ attachment site, attB, as previously described (Diederich et al., 1992). The insertion was confirmed by PCR using the following primers: 681 (5'-CGGTTGTGACGAAAGGAGC-3'), 682 (5'-TTGATGTCGATGAAAGTGC-3'), 683 (5'-TGCCCATGGGAAACAGTAGT-3') and 684 (5'-ACCACATGGTCCTTCTGAG-3').

**Western blotting.** Protein purifications were performed after static biofilm growth in Petri dishes or after biofilm growth in flow cells. The biofilm cell cultures were standardized according to optical density (OD₆₀₀ 3) and the antigen 43 (Ag43) biofilm cell cultures were standardized according to optical density (OD₆₀₀ 3). The whole chain was imaged prior to and after photo-bleaching a small area within a SAR19 biofilm cell chain for ~60 s. The fluorescence loss in photo-bleaching (FLIP) was used to study whether any lateral movement of the CFP occurred. If a neighbouring cell was completely unconnected, the level of fluorescence would be unaffected, whereas any connected cell would gradually lose fluorescence due to the lateral movement of the CFP (van Drogen & Peter, 2004).

**RESULTS**

**Cellular chain formation in *E. coli* K-12**

To study the cellular chaining phenotype, the structural maturation of SAR19 (*E. coli* CSH26Δcfp) biofilms was examined under a variety of experimental conditions using confocal scanning laser microscopy (CSLM). Various different minimal media were tested (M9, M63, FAB and MOPS supplemented with glucose), and the biofilm developmental process was studied at both 30 °C and 37 °C. Although some quantitative differences were apparent when comparing the biofilms grown in the different media, only relatively minor qualitative differences were observed (data not shown). An example of a SAR19 biofilm formed in FAB minimal medium is shown in Fig. 1(A), and a chain in Fig. 2(A) (left). Whereas the

![Fig. 1](http://mic.sgmjournals.org)
different media had only a moderate impact on the final biofilm architecture and the chaining morphology, the temperature at which the biofilms were grown had a much more obvious effect. The chaining morphology was less prominent at 30°C than at 37°C (Fig. 3A), although polar interactions were occasionally observed.

To elucidate whether biofilm cell chaining was specific to the particular E. coli K-12 studied initially, a few additional laboratory E. coli K-12 strains were tested in the flow chamber system. Several of these were found to form cellular chains; MC4100 formed very distinct chains at both 30 and 37°C, whereas cellular chain formation was much less apparent in MG1655 at either temperature (data not shown). E. coli ZK2686 did not form chains at the conditions studied here; however, it was also a very poor biofilm former (data not shown). This indicates that biofilm formation and cellular chaining are both strain- and environment-specific.

**Cellular chaining is independent of flow and substratum hydrophobicity**

Biofilm architectural changes (e.g. streamers) often arise under high shear stress conditions (Purevdorj et al., 2002; Stoodley et al., 2002). To study whether the flow conditions used in the flow chamber experiments were a prerequisite for the development of cellular chains, SAR19 was also examined under static biofilm conditions. Bacterial cellular chains did form even when SAR19 was grown statically (Fig. 1B), indicating that chaining can in fact arise under

---

**Fig. 2.** Biofilm cell chains are formed during clonal growth, but are not filamentous. (A) Photo-bleaching of the intracellular CFP in a SAR19 (CSH26cfp) biofilm cell chain. SAR19 was grown in FAB minimal medium in a flow chamber for 16 h prior to the experiment. Individual cells within the biofilm chains were bleached continuously (indicated by the white circle in this representative picture) for ~60 s (middle picture), while the entire chain was imaged to reveal if any lateral movement of CFP occurred (left, right). If one minute point in an elongated cell is photo-bleached, there will be an overall loss of fluorescence in the entire cell, whereas fully separated neighbouring cells will be unaffected. These experiments therefore clearly indicated that the chains were made up of fully separated, interacting cells. (B) Confocal scanning micrographs of a CSH26cfp/yfp flow chamber biofilm grown in FAB minimal medium. A 1:1 mixture of SAR19 (CSH26cfp) and SAR20 (CSH26yfp) was inoculated and the biofilm was grown at 37 °C. The pictures were acquired after 6 h (left-hand side) and 16 h (right-hand side) of incubation, respectively, and clearly indicate that the chains are formed by cell progeny rather than via random aggregative interactions between members of the biofilm population.
non-flowing conditions. The chains, however, were much shorter in length. They also appeared to develop mainly alongside the substratum surface with little or no extension into the surrounding environment, indicating that some spatial constraints were required. The chaining phenotype was also much less apparent at 30°C than at 37°C under static conditions (data not shown).

Some studies have suggested that the physicochemical properties of the substratum surface may also influence the biofilm architecture. A marine bacterial isolate has been shown to form long cell chains during biofilm formation when grown specifically on hydrophilic surfaces (Dalton et al., 1994). To establish whether *E. coli* SAR19 could exhibit substratum-specific biofilm development, both glass (hydrophilic) and vinyl plastic (hydrophobic) substrata were tested in the flow cell system. No significant structural changes were apparent when comparing the mature biofilms formed on the two different surfaces, indicating that the structural maturation/chaining of *E. coli* SAR19 biofilms is independent of substratum hydrophobicity (data not shown).

**Cellular chains are formed during clonal growth**

Inspection of the biofilms suggested that cell chaining was the result of multiple interacting bacteria forming chains rather than very elongated cells (Fig. 1B). Moreover, cell chains were also seen during growth in rich media, such as LB, suggesting that chain formation was not growth-related per se (data not shown). To bolster this notion, fluorescent loss in photo-bleaching (FLIP) was used. Fluorescent proteins generally diffuse freely within a bacterial cell. Hence, if one minute point in an elongated cell is photo-bleached, there will be an overall loss of fluorescence in the entire cell, whereas fully separated neighbouring cells will be unaffected (van Drogen & Peter, 2004). When individual cells within SAR19 biofilm chains were bleached, the neighbouring cells retained their initial level of fluorescence (Fig. 2A). This confirmed that the cells were completely separated.

In an effort to determine whether the cell-chaining phenotype was the result of random aggregative interactions between members of the biofilm population or whether the chains arose primarily via cell division, cfp-tagged (SAR19) and yfp-tagged (SAR20) CSH26 cells were co-inoculated and studied in the flow cell system. From these experiments it transpired that the structural maturation of CSH26 biofilms occurred by clonal growth, as individual chains were always of the same colour (Fig. 2B). The overall indication therefore was that the cell chains were formed mainly by non-dispersed biofilm progeny.
**Ag43 facilitates chain formation in E. coli K-12**

These initial experiments suggested that the cell-chaining phenotype was due to one or more surface-associated adhesive structure(s). In order to identify which adhesin(s) could be responsible, several mutants were constructed with deletions in genes encoding biofilm-related adhesins: CSH26Aflu (Ag43), CSH26csgAB (curli), CSH26fim (type I fimbriae) and CSH26cps (colanic acid). These were subsequently studied in the flow cell biofilm system. All of these mutants showed severe biofilm developmental defects when grown at 30 °C and/or 37 °C (data not shown), but only CSH26Aflu did not display any biofilm-specific cell chaining at all (Fig. 3). No structural maturation or cell chaining was seen in this mutant at either 30 °C or 37 °C, although the mutation did not affect the initial adhesion (Fig. 3A, B). MG1655Aflu had a similar phenotype (data not shown). This correlates well with the properties of Ag43, an autotransporter protein, which is known to mediate auto-aggregation, flocculation and biofilm formation (Danese et al., 2000a; Diderichsen, 1980; Klemm et al., 2004; van der Woude & Henderson, 2008). To verify that Ag43 was not simply important for the structural integrity of the biofilm, CSH26Aflu was subsequently grown statically. In the static biofilm assay, cell chancing arguably does not require any structural support, given that the chains form alongside the substratum. Under these conditions, CSH26Aflu formed no distinct cell chains at either 30 °C or 37 °C (data not shown). Finally, to complement the mutation, a vector that constitutively expresses Ag43 was introduced into the mutant strain. When complemented, the mutant strain displayed extensive cellular chancing during biofilm formation at 37 °C, clearly supporting the notion that Ag43 mediates cellular chain formation in *E. coli* K-12 biofilms (Fig. 3A, B). Interestingly, the complemented strain also formed highly elaborate chains at 30 °C. This could suggest that Ag43 was not sufficiently or continually expressed in the wild-type strain at 30 °C to allow the formation of long distinct chains. Although immunoblotting did suggest that the expression of Ag43 was somewhat lower at 30 °C than at 37 °C (Fig. 4C), Ag43 was nevertheless clearly required for the structural maturation of the biofilm.

**Cellular location of Ag43**

To verify that Ag43 was produced within the cell chains, and to study the cellular localization of this antigen, immunofluorescence microscopy was performed. While the mutant strain, and the vector control (CSH26Aflu + pACYC184), showed little or no specific immunostaining, both SAR19 and the complemented mutant (CSH26Aflu + pKKJ128) were clearly stained, confirming the expression of Ag43 within the cell chains themselves (Fig. 4A). These experiments also indicated that Ag43 was concentrated primarily at the cell poles, although some peripheral staining was often observed. This is also seen in biofilms grown at 30 °C (Fig. 4A). Most other studies so far have indicated that Ag43 is distributed uniformly on the cell surface (Henderson et al., 1997a; Owen et al., 1996). However, in the majority of these studies, Ag43 was overexpressed. This led us to speculate if the cellular distribution of Ag43 was influenced by the level of expression. To study this, the immunofluorescence experiment was repeated using an arabinose-inducible plasmid expressing Ag43. This allowed us to fine-tune the expression of Ag43 in the mutant background. As seen in Fig. 4(B), at low levels of induction, the cell chains were stained predominantly at the cell poles, while at a higher level of induction, a much more uniform distribution was observed. This indicates that overexpression does in fact influence the cellular distribution of the protein, probably due to protein ‘overflow’ to alternative sites.

**Type I fimbriae expression reduces cellular chain formation**

Type I fimbriae are known to be important during the initial stages of *E. coli* K-12 biofilm formation (Pratt & Kolter, 1998). Given that type I fimbriae (~1 μm long structures) have previously been shown to mask Ag43 functional (Ag43 protrudes ~10 nm from the cell surface) (Hasman et al., 1999, 2000; Schembri & Klemm, 2001), it was interesting to examine how type I fimbriae expression would affect cell chancing. To study this, a plasmid encoding the fim gene cluster, pPKL4, was introduced into the CSH26 wild-type strain. When overexpressed, type I fimbriae significantly reduced the formation of distinct cell chains as compared to the vector control. Some cellular chains did occasionally arise during later stages of the biofilm development (Fig. 5). This correlates well with the notion that Ag43 drives cell chancing and indicates that biofilm cell chancing can be affected by other surface structures.

**Cellular chancing in wild-type *E. coli***

To determine whether the biofilm chancing morphology was restricted to *E. coli* K-12, a range of wild-type *E. coli* were also studied in the flow chamber biofilm system. Several urinary tract infectious *E. coli* are known to undergo morphological changes when studied in a murine UTI model system (Justice et al., 2004, 2006). We therefore selected several UTI strains (Table 1), including several asymptomatic bacteriuria (ABU) strains, for this analysis. These experiments were performed in minimal medium and/or in urine at 37 °C. While the uropathogenic strains generally formed relatively flat, homogeneous biofilms in minimal medium and/or urine, the ABU isolates were much more heterogeneous. Interestingly, however, three of the ABU strains, including the prototypic ABU strain 83972, displayed a highly elaborate and loose biofilm structure when grown in urine, with long distinct bacterial cellular chains (Fig. 6). This indicates that cellular chain formation is not specific to particular *E. coli* K-12 strains, but may also be seen in bacterial UTI isolates. Interestingly, 83972 did not form chains under static conditions (in urine), indicating that in this particular strain at least, flow
shear stress may induce the formation of biofilm cellular chains (data not shown).

**DISCUSSION**

Bacterial biofilm formation is a complex and dynamic process, which can entail a wide variety of physiological events. In this work, we studied a novel structural phenotype in *E. coli* biofilms, bacterial cell chaining, and showed that cellular chain formation in *E. coli* K-12 is mediated by Ag43. Ag43 is a member of a group of self-associating autotransporter proteins, termed SAAT, which mediate auto-aggregation, flocculation and biofilm formation (Danese *et al.*, 2000a; Diderichsen, 1980; Klemm *et al.*, 2000b; Diderichsen, 1980; Klemm *et al.*, 2000b).
2006; Sherlock et al., 2004, 2005). Ag43 has also recently been implicated in the long-term persistence of a uropathogenic E. coli (UPEC) strain in the urinary tract (Ulett et al., 2007), possibly during a complex intercellular lifestyle (Anderson et al., 2003; Justice et al., 2004). The cell-chaining phenotype was not substratum-dependent, as seen in some microbial species (Dalton et al., 1994), correlating well with the implication of Ag43 in the structural maturation of E. coli biofilms rather than in the initial adhesion to the substratum. Several experimental parameters such as medium composition and temperature were examined in relation to this unique biofilm morphology. Whereas cellular chain formation was observed, at least to some extent, in all the various media studied, a much clearer difference was seen when comparing biofilms grown at 37 °C and 30 °C: the cell-chaining phenotype appeared much less distinct at 30 °C. Nevertheless, the CSH26Δflu mutant did form significantly less biofilm at 30 °C as compared to the wild-type, signifying that Ag43 is important for the structural maturation of the biofilm at both temperatures. Immunofluorescence microscopy did not indicate that the cellular localization of Ag43 changed depending on temperature. The discrepancy could lie in the complex regulation of other surface structures, possibly interfering sterically with chain formation. However, the complementation study and immunoblotting did suggest that the expression level in itself may also contribute to this macroscopic difference.

To our knowledge no evidence has been presented previously to suggest that Ag43 is polarly localized. Most auto-aggregation studies performed thus far have found a uniform distribution of autotransporters such as Ag43 and AIDA-I (Benz & Schmidt, 1992; Henderson et al., 1997b; Owen et al., 1996; Sherlock et al., 2004). However, these studies were mainly done using laboratory E. coli K-12 strains. A recent study suggested that polar localization of autotransporter proteins is a common phenomenon and that the integrity of the bacterial LPS is a key determinant controlling the final cellular localization of autotransporters; laboratory E. coli strains often have incomplete LPS (Jain et al., 2006). Many auto-aggregation studies have, however, also been based on artificially overexpressing the autotransporter proteins. Arguably, overexpression could also affect the cellular distribution of the proteins. Here we found that the cellular localization of Ag43 was in fact affected by the level of expression, most likely due to protein ‘overflow’ to alternative sites. This indicates that the cellular distribution of surface proteins may be determined by multiple factors, be it LPS and/or expression levels, depending on the strains studied. While these factors could also determine macroscopic differences, e.g. clumping versus chaining, so far we have not been able to identify any level of expression of Ag43 where there was a clear distinction between these two phenotypes. Even at the highest level of induction, where Ag43 was distributed uniformly, chains were formed in the static biofilm model. This suggests that dimensional constraints, as experienced either during growth alongside a substratum or during growth in a dynamic environment (flow), can determine the structural characteristics of a mature biofilm.

Other factors were also found to affect the cell-chaining morphology. Overexpression of type I fimbriae reduced cell chaining considerably. This correlates well with previous studies, which showed that a range of bacterial surface structures, such as fimbriae and capsule, mask Ag43 function (Hasman et al., 1999; Schembri & Klemm, 2001; Schembri et al., 2004). This could suggest that there is a coordination of type I fimbriae and Ag43 during biofilm formation, which is involved in regulating the transition between adhesion, and aggregation/chain formation.

The cellular chain formation we have observed here does not appear to be restricted to E. coli K-12, given that at least three urinary tract infectious E. coli isolates were also found to display cell chaining during biofilm formation. Interestingly, all of these were ABU isolates. Since any surface structure that shields Ag43 function would interfere with cell chaining, as demonstrated here in the case of type I fimbriae, it is not surprising that the UPEC strains we tested did not show cellular chaining. These strains are all capsule producers and also able to express many different fimbrial species, all of which would impede Ag43 function.

Biofilm chaining may therefore be more common among ABU strains, many of which lack the capacity to produce

**Fig. 5.** Type I fimbriae expression reduces cellular chaining, presumably via steric hindrance. Phase-contrast micrographs of two flow cell biofilms after 16 h growth at 37 °C in FAB minimal medium. The effect of type I fimbriae on the chaining phenotype was studied by introducing the vector plasmid pBR322 (A) or the type I fimbriae-overproducing plasmid pPKL4 (B) into CSH26.
functional fimbriae and/or capsules. The specific role of cellular chain formation, however, remains somewhat elusive. Cellular chaining, like flowing seaweed, may facilitate an increased flow of nutrients through the interior of the biofilm. Cellular chaining could also afford an increased structural coherence during high shear stress conditions. As previously mentioned, some urinary tract infectious E. coli are known to undergo significant morphological changes, such as filamentation and chaining, during colonization/invasion of the (murine) urinary tract, which is a highly dynamic environment (Justice et al., 2004, 2006). In this respect, it is interesting to note that the urinary tract isolate 83972 displayed extensive chaining under the hydrodynamic conditions studied here, but not under the static conditions, clearly indicating some differential regulation. Strain 83972 is a robust colonizer of the human bladder; it is an excellent biofilm former and is able to out-compete UPEC strains during biofilm formation (Ferrières et al., 2007). Nevertheless, it is important to note that the cellular chaining observed here appears to be very different from cellular filamentation in terms of molecular causality. Some autotransporter proteins, which are now believed to be polarly expressed, including AIDA-I, have also been implicated in cellular adherence to mammalian tissues, and a novel chain-like adherence pattern has also recently been identified in enteroaggregative E. coli (Gioppo et al., 2000; Henderson et al., 2004; Lu et al., 2006). This could suggest that polar auto-aggregative interactions may be important during the colonization of or biofilm formation in mammalian tissues. Several other microbial species have been found to form cellular chains during biofilm formation, including both Listeria monocytogenes and Serratia marcescens MG1 (Labbate et al., 2004; Rice et al., 2005; Rieu et al., 2008). The highly elaborate biofilms of S. marcescens MG1 contain both filaments and cellular chains (Labbate et al., 2004; Rice et al., 2005). Given that filamentation has previously been found to hamper grazing of swarmer cells by protozoa, it has been suggested that filamentous and/or chaining biofilms could have a protective function (Ammendola et al., 1998; Labbate et al., 2004). However, it remains to be determined whether cell chaining mediates any protection in relation to E. coli.

ACKNOWLEDGEMENTS

This work was funded by a grant from the Danish Food Agency (DFFE 3304-05-66), and the Danish Medical Research Council (271-06-0555).

REFERENCES


Edited by: J. G. Shaw