Biofilm formation by asymptomatic and virulent urinary tract infectious Escherichia coli strains

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Abstract

Escherichia coli is the most common organism associated with asymptomatic bacteriuria (ABU) in humans. In contrast to uropathogenic E. coli (UPEC) that cause symptomatic urinary tract infection, very little is known about the mechanisms by which these strains colonize the urinary tract. Here, we have investigated the biofilm-forming capacity on abiotic surfaces of groups of ABU strains and UPEC strains in human urine. We found that there is a strong bias; ABU strains were significantly better biofilm formers than UPEC strains. Our data suggest that biofilm formation in urinary tract infectious E. coli seems to be associated with ABU strains and appears to be an important strategy used by these strains for persistence in this high-flow environment.

Introduction

Many bacteria live as sessile communities adhered to surfaces, rather than as planktonic isolated cells. These compact microbial consortia, referred to as biofilms, are commonly associated with many economic and health problems (Costerton et al., 1999). In the medical field, the biofilm mode of growth has attracted particular attention, because many persistent and chronic bacterial infections are now believed to be linked to the formation of biofilms. Moreover, virtually all medical implants are prone to colonization by bacteria and these biofilms often serve as a source of recurrent infections. Bacterial biofilm infections are particularly problematic, because sessile bacteria can withstand host immune responses and are up to 1000-fold more resistant to antibiotics, biocides and hydrodynamic shear forces than their planktonic counterparts (Costerton et al., 1995, 1999).

Urinary tract infection (UTI) is a serious health problem affecting millions of people each year (Stamm & Norrby, 2001). The recurrence rate is high and often the infections tend to become chronic with many episodes. UTI usually starts as a bladder infection but often ascents to the kidneys and ultimately can result in renal failure or dissemination to the blood. UTI is the most common infection in patients with a chronic indwelling bladder catheter; bacteriuria is essentially unavoidable in this patient group (Foxman, 2002). UTI is classified into disease categories by the site of infection: cystitis (the bladder), pyelonephritis (the kidney) and bacteriuria (the urine). The colonization of urine in the absence of clinical symptoms is called asymptomatic bacteriuria (ABU). ABU occurs in up to 6% of healthy individuals and 20% of elderly individuals. As the name implies, ABU strains generally do not cause symptoms; most patients with ABU do not need treatment and in many cases the colonizing organism actually helps to prevent infection by other more virulent bacteria (Darouiche et al., 2001).

Escherichia coli is responsible for more than 80% of all UTIs and cause both ABU and symptomatic UTI (Hedlund et al., 2001). As a rule, such infections are caused by a single bacterial clone. The human urinary tract (UT) is submitted to strong hydrodynamic shear forces, and adherence to the UT epithelium enables bacteria to resist removal by urine flow and establish infection. Bacterial adherence is considered to be an essential step in the infection process and leads not only to colonization but also to invasion and host cell damage. The primary fimbrial adhesins associated with UPEC strains are type 1 fimbriae, P fimbriae and FIC fimbriae (Bäckhed et al., 2002). Relatively little is known about the mechanisms used by ABU E. coli for bladder colonization. Low abundance of adhesins could explain to a large degree why ABU stains do not cause symptoms in the host; however, it does not explain how these strains are
capable of efficient bladder colonization. Recently, we demonstrated that the ability to grow fast in urine is one possible mechanism for bladder colonization of ABU \textit{E. coli} (Roos \textit{et al.}, 2006a, c). Several studies have indicated the important role of biofilms in UTIs, notably in catheterized patients (Reid \textit{et al.}, 1992; Morris \textit{et al.}, 1999; Warren, 2001). Against this background, we have studied the biofilm-forming capacity on abiotic surfaces in human urine of a spectrum of ABU strains and compared them with that of UPEC strains.

\section*{Materials and methods}

\subsection*{Bacterial strains, fluorescent tagging and growth media}

The strains used in this study are described in Table 1. \textit{Escherichia coli} 83972, VR50 and CFT073 were fluorescently tagged by chromosomal insertion of \textit{yfp} or \textit{cfp} in the \textit{attB} attachment site of bacteriophage \textit{\lambda} as described previously (Diederich \textit{et al.}, 1992). The proper insertions were confirmed by PCR using primers 681 (5'-CGGTTTGATCA-GAAGGACG), 682 (5'-TTGATGTCGATGAAGGTGCC), 683 (5'-TGGCCATGGAACAGGTAGT) and 684 (5'-ACCA-CATGGTCTCCTTTGGAC). All cultivations were performed in Luria–Bertani (LB) or pooled human urine. Urine was collected from three to four healthy men and women, who had no history of UTI or antibiotic use in the prior two months, pooled, filter sterilized, stored at 4°C and used within the following 2–3 days.

\subsection*{Construction of plasmids}

The \textit{flu} gene encoding Ag43 was amplified by PCR and cloned into the BamHI and SalI sites of pACYC184, resulting in pPKL330. pPKL263 was constructed by cloning the PCR-amplified \textit{csgD} gene of \textit{E. coli} K-12 (MG1655) under the control of the arabinose-inducible pARA\textsubscript{BAD} promoter, between the NcoI and EcoRI restriction sites of pBAD/His A (Invitrogen).

\subsection*{Yeast agglutination assay}

The capacity of bacteria to express a \textit{\alpha}-mannose-binding phenotype, characteristic for functional type 1 fimbriae, was assayed by their ability to agglutinate yeast cells (\textit{Saccharomyces cerevisiae}) after pellicle formation in static liquid medium, which promotes type 1 fimbriation (Old & Duguid, 1970). This was performed as described previously (Roos \textit{et al.}, 2006a).

\subsection*{Motility assay}

The motility assay was used to test all ABU and UPEC strains on urine plates as described previously (Roos \textit{et al.}, 2006a). The distance of migration was measured after 16 h of incubation at 37°C. The assay was repeated three times with different batches of urine.

\begin{table}[h]
\centering
\caption{\textit{Escherichia coli} strains and plasmids used in this study}
\begin{tabular}{lll}
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\textbf{Name strains} & \textbf{Relevant characteristics} & \textbf{Reference} \\
\hline
\textbf{UPEC isolates} & & \\
1177 & O1:K1:H7 & Marild \textit{et al.} (1989)  \\
CFT073\textit{yfp} & CFT073\textit{attB::bla-rrnB1-cfp-T\textsubscript{0}} & This study \\
NU14 & O18:K1:H7 & Hultgren \textit{et al.} (1986) \\
\textbf{ABU isolates} & & \\
83972 & OR:K1:H/CO & Lindberg \textit{et al.} (1975)  \\
83972\textit{yfp} & 83972\textit{attB::bla-rrnB1-yfp-T\textsubscript{0}} & This study \\
VR50 & OR:K1:H/CO & Roos \textit{et al.} (2006a)  \\
VR50\textit{yfp} & VR50\textit{attB::bla-rrnB1-yfp-T\textsubscript{0}} & This study \\
VR89 & OR:H/CO & Roos \textit{et al.} (2006a)  \\
VR90 & O14:H/CO & Roos \textit{et al.} (2006a)  \\
VR91 & O134:H16 & Roos \textit{et al.} (2006a)  \\
VR92 & OR:H/CO & Roos \textit{et al.} (2006a)  \\
VR94 & O18acK1:H/CO & Roos \textit{et al.} (2006a)  \\
VR95 & O25:H2 & Roos \textit{et al.} (2006a) \\
VR96 & O134:H16 & Roos \textit{et al.} (2006a) \\
\hline
\textbf{Plasmids} & & \\
pKT274 & \textit{E. coli} K1 capsule operon in cosmid pHC79 & Echarti \textit{et al.} (1983) \\
pLDR8 & \lambda-Int expression vector & Diederich \textit{et al.} (1992) \\
pLDR11 & \textit{bla-attP} fragment-carrying plasmid & Diederich \textit{et al.} (1992) \\
pPKL263 & pARA\textsubscript{BAD}-\textit{csgD} in pBAD/His A & This study \\
pPKL330 & The \textit{flu} gene in pACYC184 & This study \\
pSM2361 & Cm\textsuperscript{R}, carries \textit{rrnB1}-RBSII-\textit{cfp}-T\textsubscript{0} & Reisner \textit{et al.} (2002) \\
pSM2362 & Cm\textsuperscript{R}, carries \textit{rrnB1}-RBSII-\textit{yfp}-T\textsubscript{0} & Reisner \textit{et al.} (2002) \\
\hline
\end{tabular}
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Curli and cellulose assays
The ability to express curli fimbriae was evaluated by streaking each strain on modified LB-agar plates (without NaCl) containing 0.004% Congo red (CR) and 0.002% Coomassie Brilliant Blue G. CR binding was indicated by the presence of red or pink colonies after incubation over-night at 37°C. Cellulose production was determined by streaking the strains on LB-agar plates containing 0.02% Calcofluor. The fluorescence of the colonies was checked by UV light illumination (360 nm) after overnight incubation at 37°C. Escherichia coli Nissle 1917 (Grozdanov et al., 2004) and E. coli K-12 were used as positive and negative controls, respectively.

Biofilm formation in microplates in human urine
Cells were grown overnight in pooled human urine and 15 µL was used for inoculation of 1.5 mL urine in 24-well flat-bottom microplates (Iwaki). The microplates were incubated statically at 37°C for 16 h. Adhered cells were stained with 0.1% crystal violet for 15 min, followed by washing three times with PBS. Ethanol-acetone (80:20, v/v) was added and A₅₉₀ nm was measured (Ultrospec III, Pharmacia).

Carrying capacity determination in microplates
Cells were grown in urine exactly as described in the above paragraph. After incubation, the medium in each well was pipetted thoroughly up and down to dissolve the biofilm and the final OD₆₀₀ nm was measured. Each strain was assayed in at least eight wells distributed on five different plates and the whole experiment was repeated three times in different batches of urine.

Biofilm formation in the presence of Bismuth-2,3-dimercaptopropanol (BisBAL)
BisBAL was prepared by combining Bismuth(III)nitrate (Aldrich) and 2,3-dimercaptopropanol [dimercaprol, British Anti-Lewisite (BAL), Sigma] in 1,2-propanediol (Sigma). Cells were inoculated in LB containing BisBAL (0, 1.5, 2.5, 5, 10, 15 and 30 µM) and grown statically at 37°C for 24 h. Biofilm formation was assayed as described above.

Biofilm formation in flow-cell chambers in human urine
Flow-chamber experiments were performed at 37°C in human urine, essentially as described previously (Christensen et al., 1999). Briefly, biofilms were grown on a microscope glass cover slip (Knittel 24 × 50 mm st1; Knittel Gläser) in three-channel chambers (1 × 4 × 40 mm). Each channel was inoculated with 250 µL of an overnight culture standardized to an OD₆₀₀ nm of 0.05. The cells were allowed to attach to the substratum for 1 h before the flow was turned on (3 mL h⁻¹).

Results

Biofilm formation of ABU strains in human urine is good and superior to that of UPEC strains
A spectrum of UTI E. coli strains, encompassing nine ABU and four UPEC strains, were used in this study. The nine ABU strains were randomly chosen from our strain collection while the four UPEC strains are among the most well-characterized isolates described, including the two sequenced strains CFT073 and 536 (Welch et al., 2002; Brzuszkiewicz et al., 2006). Arguably, the ability to grow in human urine must be an important criterion for colonization of the UT, and although many faecal isolates of E. coli, e.g. E. coli K-12, grow poorly or are unable to grow in human urine, all our strains grow well in urine. The biofilm-forming capacity of the strains in human urine was monitored in polystyrene microtitre plates by quantitative crystal violet staining. The ability to form a biofilm is often considered to be a virulence-associated trait. Surprisingly, it transpired that the four UPEC strains were poor biofilm formers compared with the nine ABU strains (paired two-tailed t test, P < 0.001 for all nine ABU strains against CFT073), i.e. all of the ABU strains formed significantly more biofilm than any of the UPEC strains (Fig. 1). Thus, the average ABU strain performed more than 10-fold better with respect to biofilm formation in urine than the average UPEC strain. Taken together, the data suggest that biofilm formation is an ABU-linked phenotype and presumably not directly linked to urovirulence.

Biofilm formation capacity is not a function of growth rate or carrying capacity in urine
The striking difference between the biofilm-forming capacity of our ABU and UPEC strains could be due to different growth rates in human urine. We have recently demonstrated that the model ABU strain 83972 as well as several other ABU strains are able to out-compete UPEC strains in urine (Roos et al., 2006a, c) and that the ability to compete is correlated to the growth rate (Roos et al., 2006a). Fast growth or higher carrying capacity in urine might therefore be invoked to explain the better performance of the ABU strains in biofilm formation. It transpired that there was no correlation between the growth rates of our strains and their biofilm-forming capacities (Fig. 2). In fact, among the four fastest-growing strains, there was only one top biofilm former (VR50) and among the four best biofilm formers there was only one strain, VR50, with better than average
growth rate. The final OD, or carrying capacity, was measured in identical microtitre plates and under the same conditions as the biofilm formation assay. The results revealed no positive correlation between biofilm-forming capacity and final OD. In fact, the four UPEC strains reached cell densities significantly higher than many of the ABU isolates (Table 2). Taken together, the data suggest that neither fast growth nor high carrying capacity is of prime importance for biofilm formation of UT-colonizing E. coli, and that fast growth and biofilm formation seem to be unrelated phenotypes.

Many classical biofilm-forming factors are not involved in biofilm formation by UT-colonizing strains

In order to gain a better idea of the mechanism leading to different biofilm-forming ability of the ABU and UPEC strains, we systematically investigated the major factors known to influence biofilm formation in E. coli. Previous studies have pointed out the importance of motility in biofilm formation (Pratt & Kolter, 1998). However, we found motile and nonmotile strains in both groups, suggesting that difference in motility cannot explain the enhanced biofilm-forming ability of the ABU strains (Table 2).

Fimbriae, notably type 1 fimbriae, have been implicated in biofilm formation of E. coli (Pratt & Kolter, 1998). Therefore, we probed for a possible link between fimbriation and biofilm formation. Our four UPEC strains are all able to express an impressive array of different fimbriae; nevertheless, they all perform very poorly with respect to biofilm formation as demonstrated in this work. In the ABU group, four of the nine strains, viz. VR50, VR89-92 and VR94-96, were able to express type 1 fimbriae (Table 2), as monitored by yeast agglutination following static growth condition. Meanwhile, the best biofilm-forming strain, VR89, was not able to express type 1 fimbriae, whereas the poorest biofilm former, VR90, had the capacity to make these fimbriae. Therefore, it does not seem that type 1 fimbriation is crucial for biofilm formation in UTI E. coli.
Curli and cellulose fibres are surface compounds known to improve biofilm formation in *E. coli* (Prigent-Combaret *et al.*, 2000; Re & Ghigo, 2006). When the ability of the ABU and UPEC strains to express these surface elements was checked, it transpired that no correlation appeared (Table 2); two of the best biofilm formers, ABU strains VR89 and VR92, do not produce curli or cellulose, while the UPEC strain 536 produces both and forms a very poor biofilm. In line with this, we found that when our UPEC strains were transformed with a plasmid, pPKL263, conferring constitutive overexpression of the CsgD transcriptional regulator, involved in curli and cellulose production, no increase in biofilm formation could be observed (Table 2). Finally, we analysed the effect of the expression of the Ag43 autotransporter on biofilm formation but observed no effect (Table 2); rather, biofilm formation markedly decreased in the Ag43-expressing version of the ABU strain 83972.

Altogether, the data suggest that none of these classical biofilm-associated elements can account for the difference observed in biofilm-formation capacity between ABU and UPEC strains.

**Capsule interference and biofilm formation**

Expression of bacterial capsules has been demonstrated to reduce biofilm formation due to masking of other surface structures (*Schembri et al.*, 2004; Klemm *et al.*, 2006b). UPEC strains generally make extended capsule structures. We therefore tried to reduce the capsule formation of the four UPEC strains by a capsule-interfering agent, viz. BisBAL (*Domenico et al.*, 1999). No significant increase in biofilm-forming capacity could be observed (data not shown). Meanwhile, when we introduced a plasmid carrying the full complement of *E. coli* K1 capsule synthesis genes in five selected ABU strains to permit capsule production, one of the strains, VR95, displayed a significant change in the presence of the capsule, which, surprisingly, favoured biofilm formation (Table 2). Altogether, the data suggest that the presence or absence of capsule cannot explain the difference observed between biofilm formation of UPEC and ABU strains.

**Biofilm formation under continuous flow conditions of selected ABU and UPEC strains**

In the human UT, bacteria are subjected to shear forces mediated by urine flow. In order to mimic such conditions, we proceeded to study the biofilm-formation capacity in a continuous flow chamber system using human urine as growth medium. To this end, representative strains from each group were selected, i.e. the ABU strains 83972 and VR50, and the UPEC strain CFT073. The three strains were
structures, whereas the UPEC strain performs very poorly.

Microtitre plates – the ABU strains form extended biofilm were consistent with those obtained from statically grown obtained under continuous flow conditions in human urine (Roos et al., 1992; Morris et al., 1999). Arguably, the ability to form biofilms might well be associated with virulence in UT-colonizing E. coli strains.

To monitor the biofilm-forming ability of our spectrum of UT-colonizing strains we used plastic and glass surfaces, and to mimic real-life scenarios more realistically, we used human urine as growth medium. Surprisingly, we found that urovirulence seemed to be inversely related to biofilm formation. Indeed, the biofilm-forming capacity of our randomly selected ABU strain group was markedly superior to that of the UPEC group, i.e. the average ABU strain was more than 10-fold better than the average UPEC strain in biofilm formation. Thus, judging from the present data, biofilm formation might not be a colonization strategy used by UPEC, as this faculty was virtually absent in our set of well-characterized UPEC strains. Meanwhile, the capacity to form a biofilm rather seems to be a persistence strategy in ABU strains.

UPEC strains are well equipped when it comes to fimbrial adhesins and most strains are able to express three or more different types of fimbriae (Klemm & Schembri, 2000; Roos et al., 2006a). In contrast, ABU E. coli strains have fewer or completely lack such adherence factors (Lindberg et al., 1975; Kaijser & Ahlstedt, 1977; Klemm et al., 2006a; Roos et al., 2006b). The paucity of fimbrial adhesins in ABU strains is probably closely associated with their ABU status, as fimbrial attachment to host tissues in the UT is prone to trigger symptoms in the patient (Hedlund et al., 2001; Bäckhed et al., 2002). Meanwhile, healthy adults normally produce 1–2 L of urine per day and being flushed out is a very realistic outcome for any bacterium trying to establish in the human UT (Mackintosh et al., 1975; Roos et al., 2006c). For UPEC strains that have extended armamentaria of attachment factors, this is not a problem; however, for ABU strains that are generally poorly equipped with such factors it must be a realistic possibility. We have recently demonstrated that many ABU strains grow fast in human urine (Roos et al., 2006a, c). Fast growth probably constitutes one strategy used by ABU strains for remaining in the UT. Bacterial biofilms are highly resistant to removal by liquid flow forces. Judging from the excellent biofilm-forming abilities of our ABU strains, it seems that biofilm formation constitutes an additional strategy used by ABU strains to colonize a high-flow environment like the human UT. Biofilm formation could be an important trait of ABU strains in medical settings. Thus, ABU strain 83972, which was demonstrated to be a good biofilm former in this study, has been shown to inhibit catheter colonization by uropathogens (Trautner et al., 2002, 2003). We believe that the

tagged with yfp and cfp reporter genes, resulting in strains 83972yfp, VR50yfp and CFT073cfp. The strains were inoculated in flow chambers and their ability to form biofilm on glass slide was followed over 40 h by confocal microscopy
(Fig. 3). In line with their biofilm profiles in microtitre plates, the two ABU strains, 83972yfp and VR50yfp, readily formed biofilms under these conditions, although strain 83972yfp performed somewhat better, having established a structured biofilm within 14 h (Fig. 3). Meanwhile, UPEC strain CFT073cfp performed very poorly and took 40 h to establish a few scattered microcolonies (Fig. 3). The results obtained under continuous flow conditions in human urine were consistent with those obtained from statically grown microtitre plates – the ABU strains form extended biofilm structures, whereas the UPEC strain performs very poorly.

**Discussion**

More than 50% of all microbial infections have now been associated with the formation of biofilms (Costerton et al., 1999). What make bacterial biofilms particularly troublesome from a medical perspective is the fact that biofilm formation significantly enhances resistance towards removal by both natural defence mechanisms and antibiotics (Costerton et al., 1999). Previous studies have advocated the importance of bacterial biofilm formation in UTIs, notably in chronic cystitis and infections associated with catheters (Reid et al., 1992; Morris et al., 1999).
ability of strain 83972 to inhibit catheter colonization by uropathogens must largely be due to biofilm formation.

A plethora of bacterial surface structures are known to be involved in biofilm formation. In *E. coli*, a range of surface features of both proteinaceous and polysaccharide nature have been shown to enhance biofilm formation, reviewed in (Schembri et al., 2002; Klemm & Schembri, 2004; Branda et al., 2005; Klemm et al., 2006b). To complicate matters, many of these surface structures are prone to phase variation and are therefore only expressed in a subgroup of the population. Also, as previously pointed out, extended surface structures like capsules can interfere negatively in biofilm formation by masking shorter biofilm-enhancing structures (Schembri et al., 2004). In the present study, we examined the potential role of a broad spectrum of factors that have been demonstrated to influence biofilm formation in *E. coli* as type 1 fimbriae, Ag43, curli and flagella, as well as various polysaccharide structures. As it turned out, none of these seemed to influence biofilm formation significantly in our strains. At this point, it is not clear what types of mechanisms ABU strains use that make them superior biofilm formers. Meanwhile, whatever the particular mechanism(s) in play, our observations reveal that ABU strains seem to be significantly better biofilm formers than UPEC strains. At this point, it is not clear what types of mechanisms ABU strains use that make them superior biofilm formers. Meanwhile, whatever the particular mechanism(s) in play, our observations reveal that ABU strains seem to be significantly better biofilm formers than UPEC strains, suggesting that in UT colonizing *E. coli* biofilm formation is a survival trait rather than a virulence mechanism. Also, a picture of how ABU strains manage to colonize the human UT is forming: the two primary strategies seem to be (1) fast growth and (2) biofilm formation.

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References


Biofilm formation of UTI strains


