The ferric yersiniabactin uptake receptor FyuA is required for efficient biofilm formation by urinary tract infectious *Escherichia coli* in human urine

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Urinary tract infection (UTI) is the most common infection in patients with indwelling urinary catheters, and bacterial biofilm formation is a major problem in this type of infection. *Escherichia coli* is responsible for the large majority of UTIs. Free iron is strictly limited in the human urinary tract and there is fierce competition between the host and infectious bacteria for this essential metal. Urinary tract infectious *E. coli* have highly efficient mechanisms of iron acquisition, one of which is the yersiniabactin system. The *fyuA* gene, encoding the yersiniabactin receptor, is one of the most upregulated genes in biofilm; it was upregulated 63-fold in the *E. coli* UTI strain VR50. FyuA was found to be highly important for biofilm formation in iron-poor environments such as human urine. Mutants in *fyuA* show aberrant biofilm formation and the cells become filamentous; a VR50*fyuA* mutant showed a 92 % reduction in biofilm formation in urine flow-cell chambers compared with the wild-type. The FyuA/yersiniabactin system is known to be important for virulence. Here we demonstrate a direct link between FyuA and biofilm formation in iron-poor environments. We also show that the availability of iron greatly influences UTI strains' ability to form biofilm.

Received 24 July 2007

Revised 26 September 2007

Accepted 28 September 2007

INTRODUCTION

Iron is essential for bacterial growth. Bacteria face iron-limiting conditions in the mammalian host, where free iron is strictly limited and iron is normally bound to sequestering proteins such as transferrin and lactoferrin. To counter such iron-limiting conditions bacteria use different highly efficient mechanisms of iron acquisition. A typical high-affinity iron-uptake system consists of a low-molecular-mass Fe^{3+} -chelating compound, known as a siderophore, combined with its cognate membrane-located receptor (Martinez *et al.*, 1990). Such iron-acquisition systems are generally regarded as important virulence or fitness factors. A range of enterobacteria contain a gene cluster called the high-pathogenicity island (HPI) encoding proteins for biosynthesis of the yersiniabactin (Ybt) siderophore and its uptake system (Rakin *et al.*, 1999; Schubert *et al.*, 1998, 2004). The HPI is widespread among members of the *Enterobacteriaceae* and is essential for virulence in *Yersinia* and certain pathotypes of *Escherichia coli* (Schubert *et al.*, 2004). One of the important genes residing on the HPI is *fyuA*, encoding the 71 kDa

outer-membrane protein FyuA (ferric yersiniabactin uptake), which act as a receptor for Fe-Ybt siderophore uptake (Heesemann *et al.*, 1993; Rakin *et al.*, 1994; Schubert *et al.*, 2002). The *fyuA* gene has been associated with virulence in many members of the *Enterobacteriaceae* (Schubert *et al.*, 1999, 2002).

Urinary tract infection (UTI) is a serious health problem that affects millions of people each year (Stamm & Norrby, 2001). The recurrence rate is high, and often the infections are particularly troublesome and become chronic with multiple episodes. UTI usually starts as a bladder infection but often ascends to affect the kidneys and ultimately can result in renal failure or dissemination to the blood. UTI is the most common infection in patients with indwelling bladder catheters, and bacteriuria is essentially unavoidable in this patient group (Foxman, 2002). We have recently found that two phenotypic traits are important and contribute to the urinary tract-colonizing properties of many UTI strains, namely fast growth and superior biofilm formation in urine (Hancock *et al.*, 2007; Roos *et al.*, 2006a, b).

Rather than existing as individual planktonic cells, most bacteria generally live attached to surfaces as sessile communities, also referred to as biofilms (Costerton *et al.*, 1995, 1999). Biofilms are commonly associated with many health problems (Costerton *et al.*, 1999; O'Toole *et al.*, 2000) and can form on virtually any type of surface. Bacterial biofilms have attracted particular attention,

Abbreviations: HPI, high-pathogenicity island; UPEC, uropathogenic *E. coli*; UTI, urinary tract infection.

The microarray data for this paper have been deposited in ArrayExpress (<http://www.ebi.ac.uk/arrayexpress>) with accession number E-MEXP-926.

because many persistent and chronic bacterial infections are now believed to be linked to the formation of biofilms. Also, virtually all medical implants, such as indwelling urinary catheters, are prone to colonization by bacteria and the resultant biofilms often serve as a source for recurrent infections. Bacterial biofilm infections are particularly problematic, because sessile bacteria can withstand host immune defence mechanisms and are extremely resistant to antibiotics, biocides and hydrodynamic shear forces that can efficiently clear corresponding planktonic bacteria (Costerton *et al.*, 1995, 1999). In the urinary tract bacterial biofilm-associated infections include chronic cystitis, prostatitis and catheter- and stent-associated infections (Warren, 2001). On this background we have analysed the role of FyuA-mediated iron acquisition and of iron concentration on biofilm formation of UTI *E. coli* strains in human urine.

METHODS

Bacterial strains and media. Strains VR50, 83972 and VR89 are asymptomatic bacteriuria (ABU) *E. coli* strains (Roos *et al.*, 2006a). J96, 536 and NU14 are three uropathogenic *E. coli* (UPEC) strains isolated from severe cases of UTI. Pooled human urine, or MOPS minimal medium supplemented with 0.2 % glucose (Neidhardt *et al.*, 1974), were used in all experiments. Human urine was collected from a pool of 15 healthy men and women volunteers who had no history of UTI or antibiotic use in the prior 2 months. For each experiment, urine was collected randomly from three to five of these individuals (pH 5.5–7.5). The urine was pooled (pH 6.5–7.0), filter-sterilized, stored at 4 °C, and used within the following 2–3 days. In the iron experiment, the urine was supplemented with either 10 µM FeCl₃ or 200 µM 2,2'-dipyridyl (iron chelator).

Microarray analysis. The microarray results of the yersiniabactin HPI cluster are extracted from a previously published complete genome microarray study of *E. coli* strains 83972 and VR50 during biofilm formation (Hancock & Klemm, 2007). GeneChip *E. coli* Genome 2.0 Arrays (Affymetrix) were used for hybridization of the labelled cDNA. For VR50, three chips were hybridized with samples grown in three individual flasks in MOPS medium, three chips were hybridized with samples from cells grown in pooled human urine in three individual flasks, and three chips were hybridized with samples from biofilm cells grown in pooled human urine in three individual Petri dishes. The nine VR50 arrays were normalized together and fold changes during biofilm growth in urine compared with planktonic growth in MOPS and in urine were calculated; the three arrays hybridized with samples from biofilm growth on Petri dishes were compared with the three arrays hybridized with samples from planktonic growth (i) in MOPS and (ii) in urine. In a third comparison, fold changes were calculated by comparing the arrays hybridized with samples from planktonic urine with planktonic MOPS. The supporting microarray data have been deposited in ArrayExpress (<http://www.ebi.ac.uk/arrayexpress>) with accession number E-MEXP-926.

Construction of *fyuA* knockout mutants. FyuA mutants of VR50 and 83972 were constructed using the λ Red recombinase gene replacement system (Datsenko & Wanner, 2000). Briefly, the *npt* gene from plasmid pKD4 was amplified using a primer set containing 40 nt homology extensions of the *fyuA* target gene: P707 (5'-CGCCGTCTTACAGGACTCACAACAATGAAAATGACACGGGTGTAG-GCTGGAGCTGCTTCG-3') and P708 (5'-ATCAGAAGAAATCAATTCGCGTATTGATACCGACGGTGCGCATATGAATATCCTCCT-

TAG-3'). The PCR product was transformed into host strains containing pKD46, and kanamycin-resistant colonies were selected. The λ Red helper plasmid pKD46 was cured by growth at 37 °C and the correct double-crossover was confirmed by PCR (P711, 5'-CGCCGTCTTACAGGACTC-3'; P712, 5'-CAATTCGCGTATTGATACC-3').

Construction of *fyuA* plasmid pVR3. The *fyuA* gene of VR50 was amplified by PCR (P713, 5'-GCGCCAAGCTTGTCGACCGTTATCG-CC-3' and P714, 5'-GCGCCGGATCCCGACTTTGCTGAGTTACTGG-3') containing *Hind*III and *Bam*HI restriction sites, and cloned into pBR322. The resulting plasmid, pVR3, was transformed into VR50, VR50*fyuA*, MG1655 Δ *fim* Δ *flu* and the three UPEC strains J96, 536 and NU14.

Biofilm formation in microtitre plates. Cells were pre-grown in pooled human urine and 10 µl aliquots were used to inoculate 1 ml urine in 24-well flat-bottom microplates (Iwaki). The microplates were incubated statically at 37 °C overnight and biofilm was monitored by crystal violet staining as described previously (Hancock *et al.*, 2007). Each strain was assayed in three or four wells on each plate and all experiments were repeated at least five times in different batches of urine. Two to four wells were used as blanks containing sterile urine (with metal, chelator or antibiotics when appropriate) and three or four wells were inoculated with wild-type strain for reference.

Carrying capacity and growth rate determination. Cells were grown in glass tubes containing 2 ml human urine (statically or shaking at 130 r.p.m.) and in microtitre wells statically containing 1 ml urine at 37 °C for 16 h. After overnight incubation the medium in the microtitre wells was pipetted thoroughly up and down to disperse the biofilm and the final OD₆₀₀ was measured. Each strain was assayed in three tubes and four wells in each experiment and the whole experiment was repeated three times in different batches of urine. For growth rate determination, each strain was grown in triplicate in 25 ml urine and OD₆₀₀ was measured every 30 min until stationary phase was reached.

Biofilm formation in flow-cell chambers. Flow-chamber experiments were performed at 37 °C in human urine, essentially as described previously (Christensen *et al.*, 1999). The cells were allowed to attach to the substratum for 1 h before the flow (3 ml urine h⁻¹) was turned on. Biofilm formation was monitored by scanning confocal laser microscopy (SCLM; Zeiss LSM510 microscope) using a 40×/1.3 Plan-Neofluar oil objective. The biofilm structure was visualized by fluorescent staining with SYTO9. Each strain was inoculated in duplicate channels and the whole experiment was repeated twice in different batches of urine. Biomass and percentage substratum coverage were determined using COMSTAT image-processing software (Heydorn *et al.*, 2000); Z-stacks were collected from five randomly selected regions from each independent biofilm.

RESULTS

Upregulation of *fyuA* and other HPI-associated genes

E. coli strain VR50 is a clinical urinary tract isolate; it grows well in pooled human urine and is an excellent biofilm former in this growth medium (Hancock *et al.*, 2007; Roos *et al.*, 2006a). We have recently studied the global gene expression in this strain during growth in biofilm

(Hancock & Klemm, 2007). The genomic expression profile of *E. coli* VR50 obtained during biofilm formation in urine was compared with that during exponential growth in minimal (MOPS-glucose) medium and pooled human urine. Here we focus on iron uptake genes differentially expressed in biofilm compared with planktonic growth (Fig. 1). The *c2436* gene shares 99.9% amino acid identity with the *fyuA* gene, encoding the ferric yersiniabactin uptake receptor, which has been associated with virulence in extraintestinal pathogenic *E. coli*

(Schubert *et al.*, 2002, 2004). The *fyuA* gene (*c2436*) and its flanking genes, *c2418–c2437*, were among the most upregulated in VR50 biofilm (3.1–57-fold compared with urine) and this cluster, i.e. HPI, was by far the largest contiguous upregulated gene cluster during biofilm growth. The genes in this cluster are homologous to the genes encoding Ybt biosynthesis (*c2419*, *c2424–c2434*), transport (*c2421* and *c2422*) and the transcriptional regulator (*c2423*). The expression of the biosynthetic and transport genes is repressed by iron and the ferric uptake

Fig. 1. Signal intensity of iron acquisition and uptake genes of the *E. coli* VR50 UTI strain during planktonic and biofilm urine growth. The yersiniabactin system of the HPI (*c2418–c2437*) was the only iron system upregulated during biofilm growth compared with planktonic growth in urine. *c2422*, *c2424* and *c2436* (*fyuA*) were among the ten highest upregulated genes in biofilm. *Upregulation compared with planktonic growth in MOPS minimal media. **Upregulation compared with planktonic growth in both MOPS and urine.

regulator Fur (Schubert *et al.*, 2004). *c2422* and *fyuA* (*c2436*) were respectively the second- and the fifth-highest induced genes overall during biofilm formation, and moreover, seven of the 19 genes in this cluster belonged to the 20 most upregulated in biofilm compared with planktonic growth in urine. The upregulation of these 19 genes in urine biofilm was significant compared with planktonic growth both in urine and in minimal medium, indicating a role specific for the biofilm growth mode and not dependent on urine composition. The HPI was the only iron-acquisition system upregulated in urine biofilm that was not upregulated in planktonic urine compared with MOPS minimal medium (Fig. 1). The high upregulation of this gene cluster indicates that these genes encoding biosynthesis and uptake of yersiniabactin may play a role during biofilm growth.

An *fyuA*-null mutant shows significantly reduced biofilm-forming capacity

By virtue of its high expression level during biofilm formation we surmised that the *fyuA* gene could play an important role during sessile growth. A deletion mutant, VR50*fyuA*, was made that carried a lesion of the central region of the *fyuA* gene encompassing 97 % of the gene. Growth experiments with VR50*fyuA* in pooled human urine revealed no growth limitations since the mutant strain reached the same final cell density as the wild-type parent, during both static and shaking growth conditions, and no significant difference in growth rate could be observed (0.0247 ± 0.0023 and $0.0259 \pm 0.0016 \text{ min}^{-1}$ for VR50 and VR50*fyuA*, respectively, Student's *t* test, $P=0.49$) (Fig. 2c). However, the knockout mutant showed significantly reduced biofilm formation capacity in microtitre plates (Fig. 2a). The FyuA status of the mutant strain was restored by cloning of the *fyuA* gene, resulting in plasmid pVR3, and subsequent introduction of pVR3 into VR50*fyuA*. Strain VR50*fyuA* harbouring plasmid pVR3 performed essentially like the VR50 parent with respect to biofilm formation (Fig. 2a).

The *fyuA* deletion mutant shows reduced biofilm formation in urine flow chambers

The urinary tract is a highly hydrodynamic environment with a constant flow of urine that could affect the way biofilms develop for example on artificial medical implants such as indwelling urinary catheters. Therefore, in order to mimic more realistic conditions, we investigated the ability of the *fyuA* mutant to form biofilm in a flow-chamber system using human urine as growth medium. VR50 and VR50*fyuA* were inoculated in flow chambers and the biofilm formation on glass slides was monitored over time by confocal microscopy. It turned out that, in line with the results obtained in microtitre plates, the mutant strain VR50*fyuA* was significantly impaired in biofilm formation both with respect to biomass and also with respect to biofilm structure in the flow-chamber system compared

with the wild-type strain (Fig. 3). Already after 16 h, the wild-type strain had colonized a larger proportion of the surface compared with the *fyuA* mutant. After 24 h and 40 h, the wild-type strain had covered the entire surface, while the *fyuA* mutant did not show any improvement in biofilm formation – even after 40 h it had only developed small, scattered patches on the surface. Close inspection revealed that the morphology of biofilm-associated VR50*fyuA* cells deviated significantly from that of the parent: the cells were elongated and snake-like in appearance (Fig. 3). Density scanning of the biofilms at 40 h revealed that the biofilm surface coverage and biofilm volume of the *fyuA* mutant were 15 % and 8 %, respectively, of that of the wild-type ($P<0.001$). Altogether, these results demonstrate that FyuA is required for normal biofilm formation of strain VR50 under hydrodynamic urine flow conditions, mimicking the conditions encountered in the urinary tract.

The *fyuA*-null-associated reduction in biofilm-forming capacity can be observed in other UTI *E. coli*

The well-characterized UTI *E. coli* strain 83972 is an excellent biofilm-former in human urine (Hancock *et al.*, 2007). Strain 83972 seems to employ several systems for iron acquisition when grown in urine. Global gene expression profiling has revealed upregulation of virtually all *E. coli* iron-acquisition systems when grown both *in vitro* and *in vivo*; the genes encoding enterobactin, siderophore, aerobactin and salmochelin systems, i.e. *fep*, *ent*, *fhu*, *iutA*, *iuc* and *iro*, as well as other iron-transport systems such as the *sit* and *chu* clusters, were all significantly induced in urine (Roos & Klemm, 2006; Roos *et al.*, 2006b). Also the *fyuA* gene was seen to be upregulated up to 25-fold in the human urinary tract (Roos & Klemm, 2006). In this study, a deletion mutant, 83972*fyuA*, showed a 53 % reduction in biofilm-forming capability in microtitre plates in human urine as compared with the parent strain (Fig. 2a). The result demonstrates that the *fyuA* yersiniabactin receptor-encoding gene is required for efficient biofilm formation by strain 83972 in human urine, despite the many other iron-acquisition systems expressed by the strain, and that importance of FyuA in biofilm formation is not specific for strain VR50.

Iron concentration affects biofilm formation

FyuA is the outer-membrane receptor of the Fe-Ybt siderophore, importing iron into the cells (Schubert *et al.*, 2004). The iron concentration in urine is limited, restricting bacterial activity in this growth medium. However, the ability of a UTI strain to form biofilm in urine is not directly correlated with its growth rate or carrying capacity in urine (Ferrières *et al.*, 2007; Hancock *et al.*, 2007). Therefore, three *E. coli* UTI strains were tested for biofilm formation in urine with added iron chelator at chelator concentrations that did not affect final cell

Fig. 2. FyuA influences the biofilm-forming capacity of UTI strains in microtitre plates in human urine. (a) Biofilm formation was reduced in the *fyuA* mutant of the UTI isolate VR50. The biofilm-forming capacity could be regained by the addition of iron to the growth medium or by complementation with a plasmid expressing the *fyuA* gene (pVR3). Biofilm formation was also reduced in an *fyuA* mutant of the UTI strain 83972. * $P < 0.001$. (b) Overexpression of the *fyuA* gene (pVR3) by J96, 536 and NU14 significantly increased biofilm formation of these three UPEC strains. * $P < 0.01$. All experiments (a) and (b) were performed in pooled human urine and repeated at least five times in different urine batches; error bars indicate standard deviation of five or more independent experiments. (c) Representative growth curves of VR50 (◆) and the mutant VR50*fyuA* (□) in human urine; error bars indicate standard deviation of three shake flasks.

strain VR50*fyuA* in urine with added chelator was reduced by 72 % compared with the parent in the same chelator-containing medium (Student's *t* test, $P < 0.001$), which corresponds to a reduction almost twice as large as in unmodified urine (i.e. 38 %). This indicates that the importance of FyuA in biofilm formation increases when iron becomes limited. Altogether, the results indicate that availability of iron is critical and influences the ability to form biofilm – the biofilm formation in urine increased with increasing iron concentration for a number of UTI strains, and the expression of FyuA during biofilm formation in human urine becomes more important as the availability of iron decreases.

Introduction of an *fyuA*-encoding plasmid increases biofilm formation of UPEC strains

Our results showed that an increased iron concentration resulted in better biofilm formation; we therefore wanted to investigate whether overexpression of *fyuA* by virtue of increased copy number would result in increased biofilm formation induced by increased iron uptake. When strain VR50 was transformed with pVR3 containing the *fyuA* gene a 38 % increase in biofilm formation was observed (Fig. 2a). Transformation of three well-characterized UPEC strains (which all carry the HPI), J96, 536 and NU14, with pVR3 revealed a significant increase in biofilm formation in human urine; J96, 536 and NU14 showed 70 %, 61 % and 52 % higher biofilm formation, respectively (Fig. 2b). This result indicates that iron limitation can be overcome via overexpression of an efficient iron-uptake system such as FyuA. Consequently, when UPEC strains J96, 536 and NU14 are engineered to take up iron more efficiently they are also able to produce biofilms better, despite the plasmid burden.

The FyuA protein itself does not account for improved biofilm formation

FyuA is located in the outer membrane. The FyuA protein per se could theoretically contribute to biofilm formation by altering the adhesive characteristics of the cell – for

densities in liquid cultures. Also, biofilm formation was tested in urine with added iron. It transpired that strains 83972, VR50 and VR89 all formed significantly less biofilm in the presence of iron chelator and more biofilm with iron added to the growth medium (Fig. 4). Furthermore, the reduced biofilm formation observed for the mutant strain VR50*fyuA* could be relieved by addition of iron to the growth medium (Fig. 2a), indicating that the presence of FyuA becomes less important when the availability of iron is higher. Additionally, biofilm formation of the mutant

Fig. 3. Time-course of biofilm formation by VR50 and VR50*fyuA* grown in flow chambers in urine. The *fyuA*-null mutant showed strongly reduced biofilm formation compared with the wild-type in the flow-chamber system. Biofilm development was monitored by scanning confocal laser microscopy at the indicated time points after inoculation. Vertical cross-sections through the biofilm were generated and simulated *xz* and *yz* vertical cross-sections are presented. Scale bars, 30 μm .

example by inducing autoaggregation, which is known to improve biofilm formation (Kjaergaard *et al.*, 2000; Schembri *et al.*, 2002). Therefore, a mutant version of MG1655 incapable of producing the surface elements Ag43 and type 1 fimbriae was complemented with the *fyuA* plasmid and studied with regard to biofilm formation and cell aggregation. Like other K-12 strains, MG1655 does not carry the HPI. Cells were grown under conditions (LB medium) where iron was not growth limiting. No improved biofilm formation in microtitre plates (Student's *t* test, $P=0.45$) and no cell aggregation could be observed for the FyuA-expressing cells compared with wild-type cells. Furthermore, cell aggregation properties of VR50, VR50*fyuA*, VR50*fyuA*(pVR3) and VR50(pVR3), after growth in urine and LB, were compared by settling curves and light microscopy. No differences in settling rates

and no cell aggregation were observed. The same results were seen when comparing the three UPEC strains (J96, 536 and NU14) with their respective plasmid-containing counterpart. Arguably, it is unlikely that the FyuA protein itself contributes to enhanced biofilm formation due to any autoaggregation property and independent of iron uptake.

DISCUSSION

More than 50% of all bacterial microbial infections are associated with the formation of biofilms, and artificial medical implants are particularly prone to biofilm formation (Costerton *et al.*, 1999). In the case of a high-flow environment like the urinary tract, biofilm-associated infections are particularly important, notably in infections

Fig. 4. The availability of iron greatly influences biofilm formation of the three UTI strains 83972, VR50 and VR89 in microtitre plates in human urine. Addition of an iron chelator (100 µM 2,2'-dipyridyl) or addition of iron (10 µM FeCl₃) to the growth medium significantly changes the biofilm-forming capacity in urine. * $P < 0.02$, ** $P < 0.001$. The experiment was performed in pooled human urine and repeated at least five times in different batches of urine; error bars indicate standard deviation of five or more independent experiments.

associated with urinary catheters and stents, where biofilms cause flow obstruction and serve as a source for recurrent infections (Foxman, 2002; Morris *et al.*, 1999; Reid *et al.*, 1992). However, mounting evidence supports the notion that biofilm formation also is involved in non-implant-type UTIs (Anderson *et al.*, 2003; Kumon, 2000; Soto *et al.*, 2006).

Iron is essential for both bacteria and humans, and for most infectious bacteria there is intense competition for iron with the host. The concentration of soluble forms of iron is very low in the urinary tract and is known to limit bacterial activity (Roos & Klemm, 2006; Russo *et al.*, 1999; Snyder *et al.*, 2004). Here we show that the ferric yersiniabactin uptake receptor FyuA is important for biofilm formation by urinary tract infectious *E. coli*. Our experimental conditions, using pooled human urine for biofilm formation in flow chambers, closely mimic catheterized patients. Several lines of evidence indicated the importance of the FyuA Ybt-Fe uptake receptor during biofilm formation. Firstly, global gene expression profiling of UTI strain VR50 during biofilm formation in urine showed that all genes located in the HPI were significantly upregulated compared with planktonic cells; interestingly, this was the only iron-acquisition system displaying upregulation in biofilm urine growth compared with planktonic urine that was not upregulated in planktonic urine compared with MOPS minimal medium (Fig. 1). The gene encoding the FyuA homologue was among the highest upregulated of all genes (63-fold). Secondly, an *fyuA* mutant showed significant reduction in biofilm formation compared with its parent – both on polystyrene in microtitre plates and on glass in flow chambers. However, when complemented *in trans* or

supplied with extra iron the mutant regained its biofilm-forming faculty. Thirdly, introduction of an *fyuA*-encoding plasmid into the well-characterized UPEC strains, i.e. J96, 536 and NU14, with the net effect of increasing the copy number of *fyuA* and thereby enhancing iron uptake capability, increased biofilm formation significantly. The importance of FyuA in biofilm formation was not a unique trait of strain VR50 but was also seen in another strain, 83972, in which an *fyuA*-knockout mutation had a similar, severe, effect on biofilm formation.

The majority of infectious *E. coli* strains possess multiple systems for ferric ion uptake, notably the relatively low-affinity aerobactin system and the two high-affinity systems yersiniabactin and enterobactin (Braun, 2003; Perry *et al.*, 1999; Schubert *et al.*, 1998). Our data support the notion that iron-limiting conditions affect biofilm-dwelling *E. coli* more than their planktonic counterparts. All genes located in the HPI were upregulated in urine biofilms compared with planktonic urine growth, whereas the aerobactin and enterobactin systems were not upregulated in urine biofilm compared with planktonic urine, indicating that iron uptake via yersiniabactin plays an important role in biofilm growth. HPI gene transcription is known to be strongly dependent on iron concentration (Fetherston *et al.*, 1999); so even though urine contains some iron, cells in urine biofilms must be desperate for iron. Mutant strain VR50*fyuA* did not exhibit growth limitation during planktonic growth in urine compared with the VR50 parent, whereas it showed strongly reduced biofilm formation in the same batch of urine when compared with the parent. This is probably because the prevailing conditions in biofilms with high cell density result in even fiercer competition for iron and very low local concentrations of iron. Under such conditions high-affinity iron-uptake systems like the yersiniabactin system become crucial. This does not rule out that other iron uptake systems might play a role in biofilms, but the array data indicate that the Ybt system is more 'biofilm specific' compared with other iron-related systems, which are upregulated during planktonic growth in urine as well.

The role of iron in biofilm formation has also been investigated in *Pseudomonas aeruginosa*, where iron depletion was shown to severely affect the shape and density of the biofilms (Banin *et al.*, 2005). In *P. aeruginosa* intracellular iron concentration seems to serve as a signal in biofilm formation; disruption of iron-dependent processes inhibits growth and biofilm formation (Kaneko *et al.*, 2007), and dispersal of biofilms can be induced by addition of iron chelators (Banin *et al.*, 2006). We have not observed chelator-induced biofilm dispersal in our UTI *E. coli* biofilms and our data indicate that iron starvation per se is responsible for poor growth of biofilm-associated cells. Biofilms of VR50*fyuA* not only had significantly less biomass than the parent but morphology was also affected at the cell level: cell shape became filamentous compared with the parent. Interestingly, filamentous cell shape was recently reported to be involved in UTIs in the mouse

model, and iron starvation might be involved in this phenomenon (Justice *et al.*, 2006). Recently, the importance of FyuA was underlined in an experimental vaccine study targeting pathogenic extraintestinal *E. coli*; here FyuA turned out to be one of the top candidates (Durant *et al.*, 2007).

Many bacterial diseases are associated with biofilm formation and there is mounting evidence that biofilm formation is also important in one of the top bacterial disease in humans, i.e. UTI, with more than 120 million cases per year globally. The biofilm mode of living is an important survival strategy or even in itself a virulence trait. It enhances bacterial resistance to hydrodynamic flow forces, to immune defences and to antibiotics. However, the biofilm mode of life comes at a price and in iron-depleted environments like the human urinary tract iron starvation is a very real possibility. The HPI, encoding the yersiniabactin iron uptake system, has spread through the enteric group by horizontal gene transfer. Arguably, its success in terms of high incidence speaks of high selection pressure perhaps due to a requirement for biofilm formation in iron-depleted environments.

ACKNOWLEDGEMENTS

We thank Birthe Jul Jørgensen for expert technical assistance. This work was supported by grants from the Lundbeckfonden (2711-45971) and the Danish Medical Research Council (271-06-0555).

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Edited by: B. Kenny