Biofilm exclusion of uropathogenic bacteria by selected asymptomatic bacteriuria *Escherichia coli* strains

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Many bacterial infections are associated with biofilm formation. In the urinary tract bacterial biofilms develop on both living surfaces and artificial implants, producing chronic and often intractable infections. *Escherichia coli* is the most common organism associated with urinary tract infections. In contrast to uropathogenic *E. coli* (UPEC), which cause symptomatic urinary tract infection, asymptomatic bacteriuria (ABU) strains are associated with essentially symptom-free infections. Here the biofilm-forming capacity on abiotic surfaces of selected *E. coli* ABU strains and UPEC strains in human urine was investigated. It was found that there is a strong bias for biofilm formation by the ABU strains. Not only were the ABU strains significantly better biofilm formers than UPEC strains, they were also able to out-compete UPEC strains as well as uropathogenic strains of *Klebsiella* spp. during biofilm formation. The results support the notion of bacterial prophylaxis employing selected ABU strains to eliminate UPEC strains and other pathogens in patients prone to recalcitrant infections.

INTRODUCTION

Urinary tract infection (UTI) is a serious health problem affecting millions of people each year. It is estimated that there are about 150 million cases in the world per 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 evolves to encompass 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 causes both ABU and symptomatic UTI (Ronald, 2003). Generally, such infections are caused by a single bacterial clone and are in effect

mono-cultures. Based on the symptoms they produce, UTI *E. coli* can be divided into ABU *E. coli* and uropathogenic *E. coli* (UPEC) strains.

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 medicine, biofilm-associated infections have a major impact on permanent and temporary artificial implants placed in the human body, often with devastating consequences. Moreover, biofilms associated with implants often serve as a source for recurrent infections. Many persistent and chronic bacterial infections are now believed to be linked to the formation of biofilms (Costerton *et al.*, 1995, 1999). In the urinary tract, bacterial biofilms can develop on many living surfaces and virtually all artificial implants, producing chronic and often intractable infections. Notable biofilm-associated infections include chronic cystitis, prostatitis and catheter- and stent-associated infections (Warren, 2001). Bacterial biofilms were reported to affect 90% of indwelling stents in patients (Reid *et al.*, 1992). *E. coli* is responsible for most infections in patients with indwelling bladder catheters; 10–50% of patients undergoing short-term catheterization develop UTI and essentially all patients with an indwelling urinary catheter in place for more than 30 days will have a UTI (Warren, 2001). Biofilm-associated bacteria are often hard to eradicate by antibiotics and a common observation is that soon after a course of antibiotic treatment the urinary tract will be

Abbreviations: ABU, asymptomatic bacteriuria; SCLM, scanning confocal laser microscopy; UPEC, uropathogenic *E. coli*; UTI, urinary tract infection.

readily reinfected by bacteria that survived in catheter biofilms (Warren, 2001). *E. coli* has also been reported to be able to form intracellular biofilm-like aggregates inside bladder cells making the bacteria hard to reach by both host defence mechanisms and antibiotics (Anderson *et al.*, 2003).

It is a common observation that individuals with ABU due to *E. coli* have a reduced risk of pyelonephritis if they are left untreated (Hansson *et al.*, 1989). The concept of using non-virulent but niche-dominant bacteria to prevent UTI with other, often virulent, bacteria is an example of bacterial interference. This concept can be used passively when patients infected with an ABU strain are left untreated, to prevent infections by virulent strains, or can be used actively by deliberate inoculations with selected ABU strains. One example is ABU strain 83972, which has successfully been used as a prophylactic agent to prevent infections in individuals prone to infections by harmful uropathogens (Darouiche *et al.*, 2001, 2005; Hull *et al.*, 2000; Trautner *et al.*, 2002). The mechanisms employed by such ABU strains to exclude pathogens are largely unknown. On this background we have studied the biofilm-forming capacity on abiotic surfaces in human urine of a spectrum of ABU strains and compared it with that of UPEC and UTI-associated *Klebsiella* strains. Notably, we have probed the ability of selected ABU strains to compete with uropathogenic strains during biofilm formation.

METHODS

Bacterial strains. The strains used in this study are described in Table 1. The prototypic ABU *E. coli* strain 83972 was originally isolated from a young Swedish girl (Lindberg *et al.*, 1975). VR11 and VR12 are *E. coli* asymptomatic strains isolated from different cases of ABU; the *E. coli* strain VR10 and all the *Klebsiella pneumoniae* and *Klebsiella oxytoca* strains were isolated from different cases of symptomatic UTIs.

Fluorescent tagging of strains. *E. coli* 83972, VR50 and CFT073 were fluorescently tagged by chromosomal insertion of *yfp* or *cfp* in the *attB* attachment site of bacteriophage λ as described previously (Diederich *et al.*, 1992). Briefly, the *NotI* fragment of pSM2361 and pSM2362, carrying the *cfp* and *yfp* genes, respectively, was ligated into the *bla-attP*-containing *NotI* fragment of pLDR11 and transformed into *E. coli* cells expressing λ -Int from pLDR8. Transformants were selected on ampicillin-containing plates and checked for their ability to fluoresce. The proper insertion of the fragment into the λ attachment site was confirmed by PCR using primers 681 (5'-CGGTTTGATCAGAAGGACG-3'), 682 (5'-TTGATGTCGATGAA-GGTGCC-3'), 683 (5'-TGGCCATGGAACAGGTAGT-3') and 684 (5'-ACCACATGGTCTTCTTGAG-3').

Growth media. All cultivations were performed in LB or pooled human urine. For each growth experiment, human urine was collected from three or four healthy volunteers who had no history of UTI or antibiotic use in the prior 2 months. The urine was pooled, filter-sterilized, stored at 4 °C and used within the following 2–3 days. When appropriate, antibiotics were added to the medium at the following concentrations: 100 μ g ampicillin (Ap) ml⁻¹, 50 μ g

kanamycin (Km) ml⁻¹, 50 μ g nalidixic acid (Nal) ml⁻¹ and 100 μ g streptomycin (Sm) ml⁻¹.

Biofilm formation in microtitre plates. Cells were grown overnight in pooled human urine and 10 μ l was used to inoculate 1 ml urine in 24-well flat-bottom microplates (Iwaki). The microplates were incubated statically at 37 °C overnight. Unbound cells were removed by inversion of the microplate and tapping on absorbent paper; adhered cells were then stained with 0.1 % crystal violet for 15 min. Excess stain was removed by washing with PBS. The crystal violet was then solubilized by the addition of ethanol and A₅₉₀ was measured (Ultrospec III, Pharmacia). Each strain was assayed in four wells on each plate and the whole experiment was repeated at least three times in different batches of urine. In each plate four wells were used as blanks containing sterile urine, and strains 83972 and CFT073 were inoculated in four wells each for reference.

Competition during biofilm formation in microtitre plates. The wells of a 24-well flat-bottom microtitre plate were filled with 1 ml human urine and inoculated with an equal number of cells of the two strains in competition to a final OD₆₀₀ of 0.01. After incubation at 37 °C for 24 h, the planktonic cells were removed and the wells were washed three times with 1 ml PBS. Adhered cells were carefully scraped from the wells with a pipette tip and resuspended in 300 μ l PBS, and the mixture was vortexed vigorously for 30 s to disperse cell aggregates. The detachment of cells was confirmed by staining the well with crystal violet after this treatment. The number of c.f.u. of each strain in the final biofilm population was determined by plating serial dilutions of the cell suspension onto plain LB-agar plates or LB-agar plates supplemented with the appropriate antibiotic. The initial 1 : 1 ratio was confirmed in the same way. As most of the *Klebsiella* strains tested did not have any antibiotic resistance, the difference in colony morphology was used to distinguish between *Klebsiella* spp. (white opaque colonies) and ABU *E. coli* strains (translucent colonies).

Competition during 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). Briefly, biofilms were grown on a microscope glass coverslip (Knittel 24 × 50 mm st1; Knittel Gläser) in three-channel chambers (1 × 4 × 40 mm). Each channel was inoculated with 250 μ l of a mix (final OD₆₀₀ 0.05) of the two competing strains grown overnight in human urine. 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 over time by scanning confocal laser microscopy (SCLM; Zeiss LSM510 microscope) using a 40 × /1.3 Plan-Neofluar oil objective. Acquired pictures were processed for display using the IMARIS software (Bitplane). Each competition experiment in flow chambers was performed in duplicate channels and repeated at least twice in different batches of urine.

RESULTS

ABU strains are better biofilm formers than UPEC strains in human urine

The biofilm-forming capacities of a set of UTI *E. coli* strains encompassing 11 ABU strains, randomly chosen from our strain collection, and six UPEC isolates, including the two well-characterized sequenced strains CFT073 and 536 (Brzuszkiewicz *et al.*, 2006; Welch *et al.*, 2002), were assessed in microtitre plates by quantitative crystal violet staining; human urine was used as growth medium to

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference
UPEC strains		
1177	O1 : K1 : H7	Marild <i>et al.</i> (1989)
536	O6 : K15 : H31; Sm ^R	Brzuszkiewicz <i>et al.</i> (2006)
CFT073	O6 : K2 : H1	Welch <i>et al.</i> (2002)
CFT073 <i>cfp</i>	CFT073 <i>attB</i> : : <i>bla-rrnBP1-cfp</i> -T ₀ ; Ap ^R	Hancock <i>et al.</i> (2007)
CFT073 <i>yfp</i>	CFT073 <i>attB</i> : : <i>bla-rrnBP1-yfp</i> -T ₀ ; Ap ^R	This study
NU14	O18 : K1 : H7; Sm ^R	Hultgren <i>et al.</i> (1986)
J96	O4 : K6; Nal ^R	Hull <i>et al.</i> (1981)
VR10	Cystitis isolate	This study
ABU strains		
83972	OR : K5 : H ⁻	Lindberg <i>et al.</i> (1975)
83972 <i>yfp</i>	83972 <i>attB</i> : : <i>bla-rrnBP1-yfp</i> -T ₀ ; Ap ^R	Hancock <i>et al.</i> (2007)
83972 <i>cfp</i>	83972 <i>attB</i> : : <i>bla-rrnBP1-cfp</i> -T ₀ ; Ap ^R	This study
VR11	Asymptomatic isolate	This study
VR12	Asymptomatic isolate	This study
VR50	OR : K1 : H ⁻	Roos <i>et al.</i> (2006a)
VR50 <i>yfp</i>	VR50 <i>attB</i> : : <i>bla-rrnBP1-yfp</i> -T ₀ ; Ap ^R	Hancock <i>et al.</i> (2007)
VR89	OR : H ⁻	Roos <i>et al.</i> (2006a)
VR90	O14 : H ⁻	Roos <i>et al.</i> (2006a)
VR91	O134 : H16	Roos <i>et al.</i> (2006a)
VR92	OR : H ⁻	Roos <i>et al.</i> (2006a)
VR94	O18ac : K1 : H ⁻	Roos <i>et al.</i> (2006a)
VR95	O25 : H2	Roos <i>et al.</i> (2006a)
VR96	O134 : H16	Roos <i>et al.</i> (2006a)
<i>K. pneumoniae</i>		
C105	Uropathogenic strain isolated from stool; Sm ^R	Struve & Krogfelt (2003)
i222-86	UTI isolate; Km ^R	This study
i225-86	UTI isolate	This study
i228-86	UTI isolate	This study
i239-86	UTI isolate	This study
i3-89	UTI isolate	This study
<i>K. oxytoca</i>		
i113-96	UTI isolate	This study
i127-96	UTI isolate	This study
i129-96	UTI isolate	This study
i133-96	UTI isolate	This study
Plasmids		
pLDR8	λ-Int expression vector	Diederich <i>et al.</i> (1992)
pLDR11	<i>bla-attP</i> fragment-carrying plasmid	Diederich <i>et al.</i> (1992)
pSM2361	Cm ^R , carries <i>rrnBP1-RBSII-cfp</i> -T ₀	Reisner <i>et al.</i> (2002)
pSM2362	Cm ^R , carries <i>rrnBP1-RBSII-yfp</i> -T ₀	Reisner <i>et al.</i> (2002)

better mimic *in vivo* conditions. The ability to form a biofilm is often considered to be a virulence-associated trait. However, it transpired that the UPEC strains performed poorly compared with the ABU strains (Fig. 1). The six UPEC strains tested formed 72–87% less biofilm than the prototypic ABU strain 83972. Furthermore, all the ABU strains formed significantly more biofilm than the reference UPEC strain CFT073 (paired two-tailed *t* test, *P* < 0.001). Taken together, the data suggest that among our UTI *E. coli* strain set biofilm formation is primarily an ABU-linked phenotype.

The ABU prototypic strain 83972 out-competes UPEC strains during biofilm formation

Pre-inoculation of urinary catheters with the ABU prototypic strain 83972 has previously been reported to reduce subsequent colonization with other strains significantly (Trautner *et al.*, 2002, 2003). Arguably, biofilm formation could be invoked to explain this phenomenon. To probe whether the good biofilm performance of strain 83972 would confer any advantage during mixed-strain biofilm competition in urine, equal amounts of ABU strain 83972

Fig. 1. Biofilm formation in human urine of a set of ABU *E. coli* (black bars), UPEC (white bars) and *Klebsiella* (grey bars) strains isolated from urinary tract infections. Biofilms were grown in microtitre plates and quantified by crystal violet staining. The A_{590} value for each strain is reported following standardization by the A_{590} value of the prototypic ABU strain 83972. Asterisks indicate strains that formed significantly more biofilm than the reference UPEC strain CFT073 (paired two-tailed t test, $P < 0.05$). The data shown are means of at least three independent experiments in different batches of urine; the error bars indicate SD.

and each of the four prototypic UPEC strains, NU14, J96, 536 and CFT073 cfp , were inoculated in microtitre plates. The fluorescent derivative of CFT073, CFT073 cfp , was used in place of the wild-type strain to facilitate selection; CFT073 cfp did not show any advantage/disadvantage during biofilm formation when competed against an equal number of the CFT073 parent ($48 \pm 4\%$ of CFT073 cfp was present in CFT073 cfp -CFT073 mixed biofilms). When competed against the UPEC strains it transpired that after 24 h strain 83972 accounted for more than 50 % of the total bacterial population present in each one of the four mixed biofilms (Fig. 2). The fraction of 83972 ranged from over 90 % in the case of NU14 and J96 competitors down to about 60 % in the case of strain 536. The results suggest that strain 83972 has a significant advantage over the UPEC strains when it comes to biofilm formation in urine and is able to out-perform them in this mode of growth.

Selected ABU strains out-perform a prototypic UPEC strain during biofilm formation

Having demonstrated that our ABU strains were significantly better biofilm formers than the prototypic UPEC strains, we proceeded to check whether the ability of strain 83972 to out-perform the UPEC strains during biofilm formation was restricted to this strain or whether this quality was shared with other ABU strains. We pitted three good ABU biofilm formers, i.e. VR50, VR89 and VR92, against UPEC strain NU14 (the best biofilm former of the

UPEC group). As it turned out, the ABU strains in all cases constituted more than 60 % of the biofilm population at the end of the competition, consisting of 79 %, 63 % and 76 % of the final population (Fig. 3). Interestingly, the best ABU biofilm former strain, viz. VR89, appeared to be the

Fig. 2. Results from biofilm competition between the prototypic ABU strain 83972 and selected UPEC strains after 24 h. The ABU strain 83972 and each of the UPEC strains (CFT073 cfp , NU14, J96 or 536) were mixed in human urine 1 : 1 and incubated statically at 37 °C in microtitre plates. The data shown are means of at least three independent experiments in different batches of urine; the error bars indicate SD.

Fig. 3. Results from biofilm competition between the UPEC strain NU14 and three good biofilm-forming ABU strains after 24 h. The UPEC strain NU14 and each of the ABU strains (VR50, VR89 or VR92) were mixed in human urine 1 : 1 and incubated statically at 37 °C in microtitre plates. The data shown are means of at least three independent experiments in different batches of urine; the error bars indicate SD.

less competitive strain against NU14; furthermore, its performance against NU14 was highly variable (Fig. 3). This suggests that the ability to form a monoclonal biofilm on abiotic surfaces does not directly correlate with the ability to out-compete other strains in mixed populations. Nevertheless, the data indicate that the ability to out-perform UPEC strains during biofilm formation is not a unique quality of strain 83972, but seems to be common among ABU strains.

Selected ABU strains out-compete a prototypic UPEC strain during biofilm formation in flow chambers

The urinary tract is a highly hydrodynamic environment with a constant flow of urine that could have an effect on the way biofilm develop on indwelling medical devices such as catheters. Therefore, in order to mimic more realistic conditions, we investigated the ability of the prototypic ABU strain 83972 to out-compete the prototypic UPEC strain CFT073 in a hydrodynamic flow-chamber system using human urine as growth medium. Strains 83972 and CFT073 were tagged with different fluorescent-protein-expressing genes, *yfp* or *cfp*, resulting in strains 83972*yfp*, CFT073*yfp*, 83972*cfp* and CFT073*cfp*; these were mixed in appropriate combinations and different ratios (83972 : CFT073 ratio of 1 : 1 or 1 : 10) before inoculation of the flow chambers. The 83972-CFT073 mixed biofilm was allowed to develop on a glass slide for 16 h and the proportion of each strain in the biofilm population was monitored by confocal microscopy. It turned out that, in line with the results obtained in microtitre plates, 83972*yfp* out-numbered CFT073*cfp*, even when 83972*yfp* represented only one-tenth of the initial population (Fig. 4a, b). In fact, only a few CFT073*cfp* cells

can be observed in the 83972*yfp*-CFT073*cfp* (1 : 1) mixed biofilm, where the yellow-fluorescent tagged strain, 83972*yfp*, strongly dominates and colonizes the whole surface (Fig. 4a). A similar observation was made when the strains were tagged in the reverse way, i.e. 83972*cfp* and CFT073*yfp*; in this case, the predominance of 83972*cfp* in the biofilm resulted in an essentially blue-coloured biofilm (Fig. 4c). This confirmed that the fluorescent protein used for the tagging does not affect the competitive behaviour of the strain and that 83972 out-performs CFT073 efficiently during biofilm formation in the flow-chamber system. The ability of a second ABU strain, VR50, to compete with CFT073 during biofilm formation in flow chambers was investigated. VR50 was tagged with *yfp* and the resulting VR50*yfp* strain was mixed in a 1 : 1 ratio with CFT073*cfp*. The proportion of each strain in the VR50*yfp*-CFT073*cfp* mixed biofilm was then analysed at different time points by SCLM (Fig. 4d). After 16 h, both strains had colonized the surface in similar proportions. However, after 24 h and 40 h, VR50*yfp* had clearly taken the advantage over CFT073*cfp*: VR50*yfp* covered most of the surface, while CFT073*cfp* developed small patches only. Taken together, these results demonstrate that both 83972 and VR50 ABU strains out-compete the prototypic UPEC strain CFT073 under hydrodynamic urine flow conditions, mimicking those encountered in the urinary tract and UT-associated catheters.

ABU strains out-compete *K. pneumoniae* during biofilm formation

After *E. coli*, *Klebsiella* spp. are one of the major aetiological agents involved in urinary tract infection, accounting for 6–15 % of all cases of community-acquired and hospital-acquired UTIs (Wilson & Gaido, 2004) and ranking second in certain groups of the population worldwide (Gales *et al.*, 2000; Gupta *et al.*, 2001; Yuksel *et al.*, 2006). Since little information on the biofilm-forming capacity of UTI-related *klebsiellae* is available, we tested this feature in a set of *Klebsiella* spp. strains isolated from symptomatic urinary tract infections. For this purpose, six *K. pneumoniae* and four *K. oxytoca* strains were grown in human urine in microtitre plates and the amount of attached cells was determined by crystal violet staining after 24 h. It turned out that the ability to form biofilm was very heterogeneous in this group: four of the *Klebsiella* strains (C105, i228-86, i239-86 and i129-96) appeared to be poor biofilm formers and did not perform significantly better than the worst performing UPEC strain CFT073 (paired two-tailed *t* test, $P > 0.05$); on the other hand, three *Klebsiella* strains (i222-86, i3-89 and i113-96) appeared to be excellent biofilm formers, performing up to four times better than the prototypic ABU strain 83972 (Fig. 1). Given the ability of 83972 to out-compete UPEC strains during biofilm formation, we tested whether this feature could be extended to another uropathogenic genus such as *Klebsiella* by competing 83972 against seven selected *Klebsiella* strains in mixed biofilm grown in human urine. In spite of

Fig. 4. Representative pictures of biofilm competition between the ABU strains 83972 and VR50, and the prototypic UPEC strain CFT073 under hydrodynamic flow of urine. 83972, VR50 and CFT073 strains tagged with the yellow (*yfp*) or the cyan (*cfp*) fluorescent protein were mixed in a 1 : 1 or 1 : 10 (ABU : UPEC) ratio, as indicated, and inoculated in flow chambers. Pictures of the biofilm population were acquired by SCLM. When relevant, simulated *xz*- and *yz*-vertical cross-sections are presented. The lower and right side of the *xz*- and the *yz*-section, respectively, correspond to the substratum. The scale bar represents 30 μm . (a), (b) and (c) show mixed biofilms of fluorescently tagged 83972 and CFT073 derivatives after 16 h; (d) shows the development of a VR50*yfp*-CFT073*cfp* mixed biofilm at different time points (16, 24 and 40 h post-inoculation).

the excellent biofilm-forming capacity of some of these uropathogenic *Klebsiella* strains, they were all efficiently excluded from the biofilms by strain 83972 and a majority of them constituted approximately 10 % of the population after 24 h (median = 8 %) (Fig. 5a). Interestingly, the best *Klebsiella* biofilm former (i222-86) constituted only 15 % of the final biofilm population, suggesting an absence of correlation between the monoclonal biofilm-forming capacity of a strain and its ability to compete with 83972 in the biofilm growth mode. Finally, we challenged i222-86 with the three good ABU biofilm formers out-competing CFT073, i.e. VR50, VR89 and VR92. In line with the results obtained in the ABU-UPEC co-cultures, we found that all of the ABU strains tested were able to out-number the *Klebsiella* isolate i222-86 in the biofilm population

(Fig. 5b). Taken together, our data indicate that selected ABU strains can efficiently compete against UPEC and *Klebsiella* spp. strains during biofilm formation.

Biofilm formation capacity and competitiveness are not correlated with the fitness of the strain

The differences in the biofilm-forming capacity and the ability to compete during biofilm formation of our ABU *E. coli*, UPEC and *Klebsiella* strains (Fig. 1) 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 during planktonic growth in urine (Roos *et al.*, 2006a, b) and that the ability to compete in this growth

Fig. 5. Results from biofilm competition between selected ABU *E. coli* strains and uropathogenic *Klebsiella* strains after 24 h. The ABU and *Klebsiella* strains were mixed in human urine 1 : 1 and incubated statically at 37 °C in microtitre plates. The data shown are means of at least three independent experiments in different batches of urine; the error bars indicate SD. (a) The ABU strain 83972 was challenged by four strains of *K. pneumoniae* and three strains of *K. oxytoca* isolated from patients with symptomatic urinary tract infection. (b) The three good biofilm-forming ABU strains VR50, VR89 and VR92 were used to challenge the best biofilm-forming *Klebsiella* strain i222-86.

mode was correlated with the growth rate (Roos *et al.*, 2006a). Growth characteristics in urine might therefore be invoked to explain the performance of the strains in biofilm formation. However, it transpired that there was no correlation between the growth rates of our strains and their biofilm-forming capacities (Fig. 6). Comparing the growth rates of the strains competed against each other

Fig. 6. Correlation between growth rate and biofilm-formation capacity in human urine of ABU *E. coli* (black squares), UPEC (white triangles) and *Klebsiella* (grey diamonds) strains. Values for biofilm-forming capacity are adapted from Fig. 1 and growth rates are representative values of each strain grown in human urine relative that of 83972. The lack of correlation [$r(21)=0.04$, $P>0.10$] confirms that a high growth rate does not confer better biofilm formation.

further revealed that the ability of a strain to compete during biofilm growth was not directly due to the fitness of the strain in urine; e.g. *Klebsiella* strain i222-86 displayed a growth rate similar to that of VR50 and higher than that of VR89 and VR92 although it was out-competed by them all. Also, nearly all *Klebsiella* strains reached a higher cell density in urine than 83972; C105 and i3-89 showed final cell densities over 70 % higher than 83972 but were both out-competed in the biofilm. The data suggest that fast growth is not of prime importance for biofilm formation or competitiveness of urinary tract-colonizing *E. coli* and *Klebsiella*, and that growth rate and biofilm formation seem to be unrelated phenotypes.

DISCUSSION

UTIs are microbial infections caused by a narrow and predictable spectrum of aetiological agents. Based on this observation, their treatment usually begins before any microbiological tests have been carried out and relies mostly on the empirical use of antibiotics. Meanwhile, a major problem inherent in the treatment of bacterial infections, including UTIs, is the increasing frequency of antibiotic resistance. Decades of antibiotic use have rendered many bacterial strains resistant. Consequently, the management of UTIs has become more and more difficult (Gupta *et al.*, 2001). *E. coli* and *Klebsiella* spp. are among the most common aetiological agents involved in UTI, accounting for up to 80 % and 15 %, respectively, of UTIs (Ronald, 2003; Wilson & Gaido, 2004). It seems that uropathogenic *E. coli* and *Klebsiella* are rapidly adapting to the antibiotics employed to treat UTIs. β -Lactams such as ampicillin and trimethoprim-sulfamethoxazole

(TMP-SMX) combination have for decades been considered as drugs of choice against UTIs. However, the proportion of *E. coli* and *Klebsiella* spp. strains resistant to these groups of antibiotics have increased dramatically worldwide during the 1990s (for review: Gupta *et al.*, 2001); the proportion of extended-spectrum β -lactamases (ESBL)-producing strains, principally *E. coli* and *K. pneumoniae*, increased between 1999 and 2003 among hospitalized patients in Italy (Luzzaro *et al.*, 2006); furthermore, two independent studies reported an increase of TMP-SMX resistance among UPEC isolates between 1991 and 1997 (Dyer *et al.*, 1998) and between 1992 and 1996 (Gupta *et al.*, 1999). To complicate the picture, an increasing fraction of infections are caused by multidrug-resistant strains, as highlighted in two recent studies on *E. coli*-inflicted UTI in which 22 % and 41 % of the strains were found to be multidrug-resistant (Manges *et al.*, 2001; Vranes *et al.*, 2003). Therefore, other complementary approaches are needed in order to counter this emerging problem. One interesting possibility is to use bacterial interference. Bacterial interference is based on the concept that one bacterial strain can interfere with the ability of another to colonize and infect the host. The use of commensal-type bacteria to inhibit pathogens has a large potential because such bacteria are often natural competitors of pathogens and they are easy to administer. Applications of commensal-type bacteria as probiotics have been shown to reduce the risk of infection in the gastrointestinal system and the urinary tract (Reid *et al.*, 2001). However, the mode of action employed by the commensals to out-compete the pathogens is often unknown. Here we demonstrate that biofilm competition between selected commensal-type *E. coli* and two top uropathogens, viz. UPEC strains and uropathogenic *Klebsiella* spp., might be invoked to account for the well-known ability of some ABU strains to out-compete uropathogens and to keep these out of the urinary tract (Darouiche *et al.*, 2001, 2005).

The dominance of selected ABU strains during mixed-strain biofilm competition might also be reflected in the respective proportion of the strains in the planktonic phase of the culture. Indeed, in the case of some strain combinations, the percentage of the ABU strain in the planktonic population correlated with the percentage of the strain in the biofilm population. However, this was not the general rule and in mixed biofilms consisting of VR50 plus NU14, 83972 plus i239-86 and 83972 plus i3-89 the planktonic phase contained less of the ABU strain than did the biofilm phase (data not shown), again supporting the notion that mechanisms specific to biofilm formation are involved in the ability of the ABU strains to exclude uropathogens during biofilm formation.

Implanted prosthetic devices constitute particularly attractive surfaces for bacterial colonization and biofilm formation as they have none of the protective mechanisms of mucosal surfaces, e.g. exfoliation of epithelial cells. Urinary catheters and stents are used extensively in medical settings. Between 15 and 25 % of patients in general

hospitals will have a urinary catheter in place for longer or shorter periods of time sometime during their stay (Warren, 2001). A large proportion of these patients will get symptomatic UTI because of catheter-associated bacterial biofilm formation by UPEC strains and uropathogenic *Klebsiella*. As previously mentioned, many of these strains are multidrug-resistant; however, the problem is further aggravated by the inherently high tolerance of biofilm-dwelling bacteria to antibiotics. As the name implies, ABU *E. coli* cause no or few symptoms and can coexist harmlessly with the patient for months and even years (Lindberg *et al.*, 1978). ABU strains such as the prototypic 83972 strain have been extensively used for their probiotic qualities as prophylactic agents in patients and have been found to be safe (Darouiche *et al.*, 2001; Hull *et al.*, 2000). We found that strain 83972 can out-compete a range of uropathogenic *E. coli* and *Klebsiella* strains during biofilm formation on abiotic surfaces in human urine. Arguably, this experimental scenario mimics conditions in urinary catheters and stents and might explain why deliberate inoculation of catheterized patients with 83972 can prevent subsequent infections with uropathogens. Also, several others of our ABU strains (but not all) performed excellently under these conditions when pitted against uropathogens in competitive biofilm formation. We believe that our results on the biofilm-forming qualities of selected ABU strains underline their potential as alternative treatment agents against recalcitrant uropathogens when antibiotics fail.

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