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Biofilm formation as a function of adhesin, growth medium, substratum and strain type

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ABSTRACT

Biofilm formation is involved in the majority of bacterial infections. Comparing six *Escherichia coli* and *Klebsiella pneumoniae* isolates revealed significant differences in biofilm formation depending on the growth medium. Fimbriae are known to be involved in biofilm formation, and type 1, F1C and P fimbriae were seen to influence biofilm formation significantly different depending on strain background, growth media and aeration as well as surface material. Altogether, this report clearly demonstrates that biofilm formation of a given strain is highly dependent on experimental design and that specific mechanisms involved in biofilm formation such as fimbrial expression only play a role under certain environmental conditions. This study underscores the importance of careful selection of experimental conditions when investigating bacterial biofilm formation and to take great precaution/care when comparing results from different biofilm studies.

Introduction

Rather than existing as individual planktonic cells, most bacteria 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. In the medical field, bacterial biofilms have attracted particular attention, because many persistent and chronic bacterial infections such as biliary tract infection, periodontitis, otitis media and endocarditis are now believed to be linked to biofilms. Virtually all medical implants, such as indwelling urinary catheters, are prone to colonisation 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).

The capacity of a microorganism to establish and form a biofilm on a given surface depends of the nature of the surface in question. When a surface such as a urinary catheter is exposed to complex mixtures of organic compounds found in the growth medium, various components adsorb onto the surface and form a conditioning film or substratum. This substratum essentially covers the surface and becomes the bona fide interface where microbial interaction takes place. In the case of biofilm formation, the steps are bacterial attachment, microcolony formation and build up of the biofilm. Arguably, the nature of the material constituting the surface determines the composition of the conditioning film, which in turn influences which microorganisms can attach.

Bacterial attachment to a substratum is the first step in biofilm formation (Klemm et al., 2010). It follows that bacterial adhesins are important players in initiation of biofilm formation. Various studies have probed the role of adhesins in biofilm formation. Type 1 fimbriae have been shown to play a role in biofilm formation of *Escherichia coli* (Pratt and Kolter, 1998). Likewise F1C fimbriae have been shown to be important for biofilm formation of *Escherichia coli* Nissle 1917 on glass (Lasaro et al., 2009). P fimbriae have recently been shown to promote pellicle formation, viz essentially an air-liquid biofilm (Ulett et al., 2007). However, these studies suffer from the fact that they have been performed with different combinations of substrata and growth media, and are generally not directly comparable. On this background we have carried out a systematic study where we test biofilm formation as a function of adhesins, growth media and substrata.

Materials and methods

Strains, plasmids and media

Strains 83972 and Nissle 1917 have been described previously (Grozdanov et al., 2004; Klemm et al., 2007). MG1655 used here was the wild-type, and for the plasmid-expression study MS528 (MG1655 Δ*fim*, Δ*flu*) was used (Kjaergaard et al., 2000). The

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two *E. coli* strains F-18 and 413/89-1 have been described previously (Wadolkowski et al., 1988; Wieler et al., 1996; Hancock et al., 2010b). One *Klebsiella pneumoniae* strain, i222-86, was also included (Ferrières et al., 2007a).

The plasmids used for expression of type 1 fimbriae (pPKL4), P fimbriae (pPAP5) and F1C fimbriae (pPKL143) have been described previously (Klemm et al., 2006; Roos et al., 2006a). In all cases introduction of one of these plasmids into a bacterial host gives rise to profuse fimbriation (Klemm et al., 2006; Roos et al., 2006a). The three plasmids including the control plasmid pBR322 were transformed into strains 83972 and MS528.

Pooled human urine, LB, ABTG (AB medium with 0.2% glucose and 1.0 μg/ml thiamine) and MOPS (containing 0.2% glucose) were used; ABTG and MOPS were in all cases supplemented with 0.02% casamino acids. Urine was collected from a pool of ten healthy women volunteers who had no history of UTI or antibiotic use in the prior two 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 sterilised, stored at 4 °C, and used within the following two to three days.

Analysis of fimbriae expression

The capacity of bacteria to express a D-mannose-binding phenotype, characteristic for functional type 1 fimbriae, was assayed by their ability to agglutinate yeast cells (*Saccharomyces cerevisiae*) on glass slides. Aliquots of bacterial suspensions (10 μl) transformed with pPKL4 and control cells transformed with pBR322 were mixed with yeast cells (10 μl) and agglutination was investigated.

The ability of cells to express P fimbriae was assayed by haemagglutination with human-type A red blood cells (RBCs). RBCs were washed three times with phosphate-buffered saline (PBS) and resolved in PBS to 5% RBCs. Aliquots of bacterial suspensions (10 μl) transformed with pPAP5 and control cells transformed with pBR322 were mixed with RBCs (10 μl) on glass slides and haemagglutination was investigated.

The ability of cells to express F1C fimbriae was assayed by agglutination with anti-F1C fimbriae serum. Aliquots of bacterial suspensions (5 μl) transformed with pPKL143 and control cells transformed with pBR322 were mixed with anti-F1C fimbriae serum (5 μl) on glass slides and agglutination was investigated.

Growth rates in microtitre plates

Cells were pre-grown in ABTG, MOPS, urine and LB, and 750 μl of fresh media was inoculated to an OD_{600} of 0.05 in 48-well flat-bottom microplates (Iwaki). The microtitre plate was incubated statically at 37 °C and the plate was scanned every 30 min at A_{595}. Each strain was grown in six wells per plate. Growth curves for each strain in each media were calculated from means of six wells and growth rate constants (μ) were calculated from three (urine) or four (LB, ABTG and MOPS) time points ($R^2 > 0.995$) of exponential growth near the start of the cultivation.

Biofilm formation in microtitre plates

Cells were pre-grown in ABTG, MOPS, urine and LB, and 10 μl were used to inoculate 1.0 ml of growth medium in 24- or 48-well flat-bottom microplates (Iwaki). The plates were incubated statically at 37 °C for 16 h and biofilm was monitored by crystal violet staining (Hancock et al., 2007). Each strain and construct was assayed in three to four wells per plate, and experiments were repeated at least five times. Ampicillin was added to wells with plasmid-containing constructs and to sterile media for use as a comparable background.

Biofilm formation in glass tubes

Cells were pre-grown in ABTG, urine and LB, and 20 μl were used to inoculate 2.0 ml of fresh growth medium in 10-ml glass tubes. The tubes were incubated statically or shaking at 130 rpm at 37 °C for 16 h and biofilm was monitored by crystal violet staining (Ferrières et al., 2007b). Each strain and construct was assayed in three tubes in each experiment, repeated at least three times. Ampicillin was added to tubes with plasmid-containing constructs and to sterile media for use as a comparable background.

Statistical analysis

Comparison of the biofilm-forming capacity of the strains was evaluated using one-way ANOVA, $P < 0.05$. Biofilm formation of plasmid-containing fimbria-expressing strains compared with plasmid-containing control strains was evaluated using paired *t* test, $P < 0.05$.

Results

Biofilm formation is significantly dependent on growth media

In order to investigate the influence of growth media on biofilm formation, six strains were selected: five *E. coli* strains including a commensal intestinal isolate (F-18), a probiotic urinary tract isolate (83972), a probiotic intestinal isolate (Nissle 1917), a K-12 lab strain (MG1655) and a pathogenic enterohaemorreagic isolate (413/89-1), and one symptomatic UTI *K. pneumoniae* isolate (i222-86). Biofilm formation was examined in microtitre plates in four different media, i.e. two different minimal lab media (ABTG and MOPS both including casamino acids), pooled human urine and LB medium. All isolates grew well in all four media. Comparing biofilm formation in the four media for each isolate revealed that 83972 made significantly more biofilm in LB compared with the other three media whereas i222-89 made most biofilm in human urine, (one-way ANOVA, $P < 0.05$) (Fig. 1a). Nissle 1917 and F-18 performed best in MOPS minimal medium. Overall, the pathogen 413/89-1 was a poor biofilm former and made the least biofilm of the six strains investigated in all four media.

Previous studies have shown that strain 83972 is an excellent biofilm former in human urine (Hancock et al., 2007; Hancock and Klemm, 2007; Hancock et al., 2008). In line with this, comparison of biofilm formation of the different isolates with that of 83972 revealed that 83972 formed significantly more biofilm than five and four of the isolates in LB and human urine, respectively – it was outperformed only by the other urinary tract isolate, viz. i222-86, in human urine. However, three isolates made more biofilm than 83972 in both minimal media, i.e. Nissle 1917, 125% and 177%; F-18, 190% and 364%; and i222-86, 358% and 412% of that of 83972 in ABTG and MOPS, respectively (one-way ANOVA, $P < 0.05$) (Fig. 1a). Interestingly, the four intestinal isolates performed rather well in human urine. They all formed biofilms that were 37–77% of that of the good biofilm former 83972, which is significantly more biofilm than that of several UPEC strains (forming 4–11% of that of 83972) under identical conditions, i.e. same microtitre plates, incubation time and medium (Hancock et al., 2007).

In previous reports studying various *E. coli* isolates no correlation between growth rates and biofilm formation was observed (Ferrières et al., 2007a; Hancock et al., 2007, 2010b). Likewise, comparing biofilm-forming capacity and growth rate of the six strains in the four different media showed no significant correlation between the two faculties (Fig. 1b).

Taken together, the results clearly revealed that the growth medium significantly influenced biofilm formation; all six strains