Biofilm formation by Acinetobacter baumannii strains isolated from urinary tract infection and urinary catheters

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Keywords
biofilm; cell surface hydrophobicity; chromosomal DNA transformation assay; lectins; plasmid curing; urinary tract infections.

Abstract
Fifty Acinetobacter isolates were obtained from urinary tract infections and urinary catheter samples. Analytical profile index assays identified 47 isolates as Acinetobacter baumannii and three as Acinetobacter lwoffii. Six A. baumannii isolates (A1–A6) displayed hydrophobicity indices >70%. Twenty isolates exhibited lectin activity. Biofilm formation by these isolates was compared with those with low hydrophobicity index values (A45–A50). Biofilms on different surfaces were confirmed by light microscopy, epifluorescence microscopy and by obtaining scanning electron microscope images. Biofilm production was maximal at 30°C, pH 7.0 in a medium with 5.0 g L⁻¹ NaCl, and its efficiency was reduced on urinary catheter surfaces at sub-minimum inhibitory concentration concentrations of colistin. Plasmid-mediated antibiotic resistance was observed in selected isolates of A. baumannii and experiments of conjugation and transformation showed the occurrence of gene transfer. Plasmid curing was used to examine the function of plasmids. Five plasmids of A. baumannii A3 were cured but no differences were observed between wild-type and plasmid-cured strains with respect to the biofilm formation capabilities. The prevalence of A. baumannii strains with biofilm mode of growth could explain their ability to persist in clinical environments and their role in device-related infections.

Introduction
The genus Acinetobacter includes a group of bacteria that are nonmotile, Gram-negative cocccobacilli, displaying strict aerobic metabolism. Acinetobacter spp. have evolved as important nosocomial pathogens. They are found in diverse environments such as soil, water, food products and are often isolated from medical devices (Bergogne-Bézáín & Towner, 1996). They cause severe infections in immune-compromised patients by colonizing on different medical devices and surviving on these surfaces (Tomaras et al., 2003). A large number of reports describe the outbreaks of Acinetobacter-associated nosocomial infections such as secondary meningitis, pneumonia, wound, burn and urinary tract infections (UTI) (Bergogne-Bézanin et al., 1993; Patwardhan et al., 2008).

Biofilm formation is an important feature of most clinical isolates of Acinetobacter spp. Biofilms are assemblages of surface microbial cells that are enclosed in an extracellular polymeric matrix (Donlan, 2002). It is clear from the epidemiologic evidence that Acinetobacter biofilms play a role in infectious diseases such as cystic fibrosis, periodontitis, in bloodstream and UTI because of their ability to indwell medical devices (Struelens et al., 1993; Donlan & Costerton, 2002; Gaddy et al., 2009). Acinetobacter is known to show resistance to a majority of commercially available antibiotics (penicillins, aminoglycosides, cephalosporins, quinolones) and therefore raises an important therapeutic problem (Smolyakov et al., 2004; Shin et al., 2009). A control of the spread of these infections thus demands the removal of Acinetobacter spp. from medical settings (Zavascki et al., 2010). Antibiotic resistance markers are often plasmid borne and plasmids present in Acinetobacter strains can be transferred to other pathogenic bacteria (Chopade et al., 1985; Patwardhan et al., 2008). The ability...
of Acinetobacter species to adhere to the surfaces, form biofilms, display antibiotic resistance and gene transfer means that there is an urgent need to study the factors responsible for their spread.

In the present study, biofilm formation on different abiotic surfaces by six clinical isolates of Acinetobacter baumannii obtained from UTI, as well as catheter surfaces, and the effects of physical parameters (temperature, pH and NaCl) on biofilm formation, was investigated. Factors such as cell surface hydrophobicity (CSH) and production of lectins, important in biofilm formation, were also evaluated. The effect of different antibiotics on Acinetobacter isolates was determined. The role of plasmids in antibiotic resistance was evaluated by plasmid curing and gene transfer experiments. The genetic and molecular analysis of these factors could explain the resistance and survival of this opportunistic pathogen under adverse conditions such as those found in patients and nosocomial environments and could prove the significance of biofilm formation and antibiotic resistance in UTI-associated Acinetobacter isolates.

Materials and methods

Isolation and identification of Acinetobacter spp.

Urine samples and urinary catheters from patients with UTI were collected from two hospitals in Pune, India using standard procedures. The samples were collected aseptically and isolation was performed on selective Acinetobacter baumannii minimal medium (Juni, 1972), cystein lactose electrolyte deficient agar (HiMedia, Mumbai), Holton’s medium (Holton, 1983) and violet red bile agar (HiMedia). UTI samples were used and transferred to sterile medium. The biomass media, while the urinary catheter surfaces were scraped suitably diluted and plated onto the selective agar plates. The plates were incubated at 37 °C for 24 h. Fifty strains of Acinetobacter spp. were identified at genus level based on their morphological characteristics and modified chromosomal DNA transformation assay (Yovan-kar et al., 2007). The biochemical characterization and identification of these isolates at genus and species levels was confirmed using the analytical profile index (API) assays (BioMerieux, Marcy l’Etoile, France). API ID32GN is a standard system equipped with 32 miniaturized assimilation tests with a computerized database for Gram-negative bacteria and different clinical Acinetobacter isolates (Towler & Chopade, 1987). The identified isolates were stored in glycerol stock at −80 °C. The bacterial isolates were inoculated in Luria–Bertani (LB) broth, incubated at 37 °C for 24 h and used for further experimentation.

Bacterial CSH

CSH was determined by the affinity test to xylene (Teixeira et al., 1993). The hydrophobicity index (HI) was calculated using the following equation:

\[
HI = \frac{(A_{600 \text{nm}} - B_{600 \text{nm}})}{A_{600 \text{nm}}} \times 100,
\]

where \(A_{600 \text{nm}}\) denotes the initial absorbance and \(B_{600 \text{nm}}\) represents the absorbance after vortex mixing. The isolates were considered as strongly hydrophobic when the HI was > 70% and with hydrophilic character when the HI was < 30% (Jones et al., 1996). Results were statistically analyzed using the Student’s t-test at a 5% significance level.

Screening of lectin production in biofilm-forming isolates of A. baumannii

The biofilm-forming isolates of A. baumannii were grown in LB at 30 °C for 24 h. The bacterial suspension was centrifuged at 6000 g at 4 °C for 40 min. Fresh human blood was washed three times with sterile normal saline. Saline and 3%/v/v human erythrocytes (50 µL each) were added to 100 µL of bacterial supernatant in each well of the microtiter plate and mixed by rotation for 5 min. Normal saline and uninoculated LB were used as the negative controls and phytohemagglutinin was used as the positive control. Agglutination of RBCs was determined within 30 min to 1 h (Patil & Chopade, 2001). The agglutinated cells were scored as positive for the presence of lectin.

Biofilm formation on abiotic surfaces

Acinetobacter isolates were inoculated in LB broth and incubated overnight at 37 °C.

Polypropylene surfaces

After the incubation period, 0.1 mL of the culture was added to 10 mL LB (0.5 × ) and dispensed in 20-mL polypropylene centrifuge tubes. The experiments were performed in duplicates and the cultures were incubated at 37 °C for 72 h under two sets of different conditions: (1) shaking at 200 r.p.m. and (2) stationary. Biofilms were quantified using the standardized crystal violet method (OToole et al., 1999; Dusane et al., 2008a).

Microtiter plates (polycarbonate)

Adhesion of bacteria to 96-well polycarbonate microtiter plate surfaces was carried out by inoculating 20 µL of overnight grown culture in 0.5 × LB containing 180 µL of the growth medium. The plates were incubated at 37 °C for 72 h and biofilm formation was estimated by a routine crystal violet staining method (Dusane et al., 2008b). The experiments were carried out in triplicates.
Glass surfaces

Biofilm formation was also analyzed in glass test tubes (Tomaras et al., 2003). The biofilms were formed by adding 0.1 mL of the culture to 10 mL LB (0.5 × ) dispensed in glass test tubes. The experiment was performed in duplicates and the cultures were incubated at 37 °C for 72 h under two sets of different conditions: (1) shaking at 200 r.p.m. and (2) stationary. After incubation, the medium was removed, the tubes were washed with distilled water, air dried and biofilms were assayed using the crystal violet method. Strains of Escherichia coli HB101 and Pseudomonas aeruginosa PA01 were used as controls for the biofilm experiments (Kazemi-Pour et al., 2007).

Bacterial adhesion to urinary catheters and effect of colistin antibiotic

In vitro assay of bacterial adhesion to the catheter surface was assessed as described earlier with some modifications (Sheth et al., 1983). The selected isolates used for this study were cultivated for 24 h at 30 °C in 0.5 × LB containing 0.25 × minimum inhibitory concentration (MIC) (0.5 μg mL⁻¹) and 0.5 × MIC (1 μg mL⁻¹) concentrations of colistin (Sigma, India). After the incubation period, antibiotic was removed from the culture by rinsing twice with sterile saline followed by centrifugation (6000 g for 10 min). The bacterial cells were resuspended in sterile saline and the OD of each suspension was measured colorimetrically at 540 nm to achieve the cell density equivalent to 1–5 × 10⁷ CFU mL⁻¹ (confirmed by plate count). Cultures without antibiotics were used as the controls. Urinary catheters (Rusch GmbH, Kemen, Germany), 7 mm in diameter were cut into 1.5-cm-long segments. The segments were then immersed in 13 × 100 mm tubes containing suspensions of the previously standardized strains and kept at room temperature for 30 min. After this contact, each fragment was placed in a tube (18 × 160 mm) containing 15 mL of sterile saline solution, and the tubes were manually inverted 40 times. This procedure was repeated 15 times, transferring the fragment to 15 tubes successively, with the objective of removing the nonadherent bacteria. After the 15 rinses, the catheter fragments were removed from the tube and rolled over the surface of 10 Petri dishes (90 × 15 mm) containing LB agar. After an incubation period of 24 h at 30 °C, the bacterial colonies were counted. The number of colonies indirectly showed the number of bacteria that adhered to the catheter surface. The result was represented as log₁₀ of number of bacteria that adhered to the catheter surface using the following formula:

\[ \text{Catheter surface area} = \pi \times \text{diameter} \times \text{length of catheter} \]

The reduction percentage in the number of adhered bacteria to the catheter was calculated for all cultures treated with antibiotics using the mean count of the control culture as reference (100%). Three duplicate experiments were carried out for each bacterial isolate. The data were compared by Student’s t-test at a 5% significance level (Costa et al., 2006).

Effect of environmental conditions on biofilm formation by selected A. baumannii strains

The effect of pH, temperature and salt concentration on biofilm formation by six A. baumannii isolates displaying high HI values and producing lectins (designated as A1, A2, A3, A4, A5 and A6) were assessed in 96-well microtiter plates. The extent of biofilms formed by A. baumannii were analyzed in the range of pH 4.0–8.0, temperature of 4, 20, 30 and 37 °C, and salt concentrations of 0%, 0.5%, 1.0%, 2.0%, 3.0% and 4.0% (w/v NaCl). Cultures of A. baumannii (20 μL) were cultivated for 24 h and added to each well of the microtiter plate containing 180 μL of LB. The plates were incubated at 30 °C for 72 h. The extent of biofilm formation was estimated using the crystal violet method as mentioned earlier (Pruthi et al., 2003; Dusane et al., 2008a).

Microscopic analysis of biofilms

The biofilm formation ability of different cultures on a variety of surfaces was visualized by light microscopy (Lawrence and Mayo, India), epifluorescence microscopy (Leica, Germany) and scanning electron microscopy (Joel, Japan). Briefly, A. baumannii biofilms were formed on glass and polycarbonate surfaces and observed under a light microscope after staining with 0.1% w/v crystal violet for 5 min (Tomaras et al., 2003). Epifluorescence microscopic examinations of the biofilms were made after staining with 0.02% acidic orange for 5 min. The excess stain was washed and biofilms were observed under epifluorescence microscope with UV filter at 400–450 nm emission wavelength. Scanning electron microscopy (SEM) (Analytical SEM; Jeol, JSM-6360-A) analysis was done according to the methodology established (Tomaras et al., 2003; Dusane et al., 2010), with some modifications. The biofilms were formed on glass and polycarbonate surfaces under static growth conditions for 72 h at 30 °C. Biofilm formation on urinary catheters (Rusch GmbH; 1 cm size) was also evaluated. The cultures were grown overnight with shaking at 30 °C and urinary catheters were added to the tubes and kept on the shaker at 30 °C for 3 days with replacement of culture medium at 24-h intervals. After biofilm formation, surfaces of the glass, polycarbonate and catheter were washed with sterile phosphate-buffered saline (PBS) and fixed with 4% v/v glutaraldehyde in 0.2 M PBS (Dusane et al., 2010).
Antibacterial susceptibility test

The susceptibility of six biofilm-forming isolates of A. baumannii to 27 antibiotics (HiMedia) from different groups was investigated out on Mueller–Hinton agar (HiMedia) using the Kirby–Bauer disc diffusion method. Discs were checked for efficacy against standard strains recommended by the National Committee for Clinical Laboratory Standards (1997) as well as others with known antibacterial susceptibility pattern. Results were interpreted as percent sensitive (%S), percent resistant (%R) and percent intermediate (%I) (Pardesi et al., 2007).

Determination of MIC of antibiotics

Determination of the MIC required to inhibit the growth of six strains of A. baumannii using 14 antibiotics from different groups were carried out by an agar dilution method (Deshpande et al., 1993). Antibiotics were checked in the range of 1–1024 μg mL⁻¹ (National Committee for Clinical Laboratory Standards, 2000).

Bacterial plasmid isolation and molecular weight determination

Plasmid isolation was done using the O’Sullivan and Klaenhammer method (O’Sullivan & Klaenhammer, 1993). Agarose gel electrophoresis was performed by 0.8% w/v agarose gel prepared in Tris-acetate buffer. Plasmid profiles were documented under UV light in gel documentation system (Alpha Innotech Corp.). Molecular weights of plasmids from different A. baumannii isolates were determined using the molecular weight determination parameter in gel documentation system (Alpha Innotech Corp.). The plasmids from E. coli V517 (MTCC 131) were also included as the positive controls and used for comparison to test plasmids as well as molecular weight determination (O’Sullivan & Klaenhammer, 1993).

Plasmid curing

Multiple plasmid-containing A. baumannii strains (A1, A2 and A3) with biofilm formation ability were selected for plasmid curing using E. coli MTCC 131 as a standard control. Curing was performed by the use of different curing agents such as ethidium bromide, plumbagin, acriflavin and acridine orange (Shakibaie et al., 1999). The percentage of curing efficiency was expressed as the number of colonies with cured phenotype per 200 tested colonies. The confirmation of cured clones was performed by agarose gel electrophoresis. The MIC of cured colonies was also tested for loss of resistance to antibiotics by an agar dilution method (Shakibaie et al., 1999; Cusumano et al., 2010).

Gene transfer from biofilm-forming A. baumannii to other bacteria

Conjugational gene transfer was performed from A. baumannii A3 pUPI 801–807 (A', Cu', Gi', Ca', Cp', Nf') to E. coli HB 101 (rifampicin-resistant mutant) by the membrane filter technique (Chopade et al., 1985). The frequency of intergeneric conjugation was determined as the number of transconjugants obtained mL⁻¹ on selective medium divided by total viable count of the recipient (Deshpande & Chopade, 1994).

Natural transformation was performed using the plate assay (Ray & Nielsen, 2005). Acinetobacter baylyi 7054 trpE was used as the host for transformation experiments and plasmid DNA from A. baumannii A3 was prepared as the donor strain (O’Sullivan & Klaenhammer, 1993). The experiments were carried out using plasmids: pUPI 801–807 (A', Cu', Gi', Ca', Cp', Nf') from A. baumannii A3 and competent cells of A. baylyi 7054 trpE as the recipient. They were confirmed for the presence of transferred plasmids according to O’Sullivan & Klaenhammer (1993). MIC values of transformants were determined by an agar dilution method and compared with the donor strain. Transformation efficiency was calculated as number of transformants μL⁻¹ of plasmid DNA (Patwardhan et al., 2008).

Results

Isolation of Acinetobacter spp. from UTI and urinary catheter samples

An API assay identified 44 isolates obtained from urine samples as A. baumannii and three as Acinetobacter lwoffi, while urinary catheter samples yielded three A. baumannii isolates.

Bacterial CSH

CSH indices for all the 50 isolates of Acinetobacter obtained from UTI and urinary catheters were determined and they varied from 34% to 79.4%. Nine strains had an HI value between 30% and 40%; six isolates displayed HI values between 41% and 50%; for seven isolates the HI values were between 51% and 60%. For the majority of the strains (22), the HI values varied between 61% and 70%. The six strains of A. baumannii (A1, A2, A3, A4, A5 and A6) that showed the highest hydrophobicity indices are listed in Table 1. Six isolates with the lowest HI values (A45–A50) were also selected. Escherichia coli HB101 and P. aeruginosa PA01 were used as the negative and positive control cultures, respectively. The difference between the six strains with the highest HI values and six with the lowest HI values was found to be significantly different with P < 0.05.
Lectin production by biofilm-forming strains of *A. baumannii*

Twenty isolates displayed lectin activity while the remaining 30 did not. A1–A6 produced lectins and A45–A50 did not. Figure 1a shows the HI values of the six strains that produced lectins and displayed the highest HI values (A1, A2, A3, A4, A5 and A6). These values were compared with those strains that had the lowest HI indices and did not produce lectins (designated as A45, A46, A47, A48, A49 and A50). Standard lectin (phytohemagglutinin) displayed hemagglutination, while normal saline and uninoculated LB used as negative controls did not show any reaction.

Biofilm formation by *Acinetobacter* isolates

The biofilm formation abilities of all the 50 isolates were determined. Quantitative analysis of biofilms formed by *A. baumannii* on glass and polypropylene surfaces showed that shaking conditions were suitable for biofilm formation. The biofilm formation by strains of *Acinetobacter* with high hydrophobicity (A1–A6) was higher and significant difference was observed compared to strains to low hydrophobicity (A45–A50) with less biofilm-forming ability with \( P < 0.001 \) (Fig. 1b). Adhesion of *A. baumannii* on polypropylene was higher than on glass surfaces (Fig. 2). Figure 1 depicts biofilm formation by a representative *A. baumannii* isolate (A3). The biofilm formation by *P. aeruginosa* PAO1 was found to be similar to that of *A. baumannii*, while *E. coli* was ineffective in forming biofilms on these surfaces (results not shown).

Effect of environmental conditions on biofilm formation by *Acinetobacter* isolates

Biofilms of six *A. baumannii* isolates were formed optimally at 30 °C, at pH 7.0 and when supplemented with 5.0 g L\(^{-1}\) NaCl. The results of *A. baumannii* A3 were shown as a representative isolate.

Microscopic experiments

Light microscopic examination of biofilm-forming *A. baumannii* cells attached to the polycarbonate and glass surfaces were performed and quantified with crystal violet. The biofilms formed on polycarbonate surfaces were more extensive than those on glass. Similar results were obtained when biofilms of *A. baumannii* were stained with acridine orange and examined under the epifluorescence microscope (Fig. 3). SEM analysis of biofilms formed by the representative *A. baumannii* A3 revealed that the cells were linked to each other by means of a dense extracellular polymeric substance (Figs 4 and 5).

Antibiotic susceptibility patterns

The six *A. baumannii* biofilm-forming isolates were tested for resistance or sensitivity to 27 antibiotics from different groups. All strains were sensitive to colistin. The resistance pattern of isolates was 83.3% for β-lactams, 94.4% for hydrophobicity (A1–A6) was higher and significant difference was observed compared to strains to low hydrophobicity (A45–A50) with less biofilm-forming ability with \( P < 0.001 \) (Fig. 1b). Adhesion of *A. baumannii* on polypropylene was higher than on glass surfaces (Fig. 2). Figure 1 depicts biofilm formation by a representative *A. baumannii* isolate (A3). The biofilm formation by *P. aeruginosa* PAO1 was found to be similar to that of *A. baumannii*, while *E. coli* was ineffective in forming biofilms on these surfaces (results not shown).

**Table 1.** HI of the cellular surface determined by the microbial adhesion method to xylene

<table>
<thead>
<tr>
<th>Strains</th>
<th>HI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. baumannii</em> A1</td>
<td>72.00</td>
</tr>
<tr>
<td><em>A. baumannii</em> A2</td>
<td>79.20</td>
</tr>
<tr>
<td><em>A. baumannii</em> A3</td>
<td>79.40</td>
</tr>
<tr>
<td><em>A. baumannii</em> A4</td>
<td>70.29</td>
</tr>
<tr>
<td><em>A. baumannii</em> A5</td>
<td>71.97</td>
</tr>
<tr>
<td><em>A. baumannii</em> A6</td>
<td>71.27</td>
</tr>
<tr>
<td><em>P. aeruginosa</em> PAO1</td>
<td>73.34</td>
</tr>
<tr>
<td><em>E. coli</em> HB101</td>
<td>8.00†</td>
</tr>
</tbody>
</table>

\[ HI = \frac{(A_{600\text{nm}}-B_{600\text{nm}})}{A_{600\text{nm}}} \times 100/A_{600\text{nm}}. \]

Controls:


*HI values significantly different with \( P < 0.05 \) than *Acinetobacter* strains studied.*

**Fig. 1.** Representative strains of *Acinetobacter baumannii* with the presence and absence of lectin showing HI (a) and biofilm formation (b) on polycarbonate (□), polypropylene (▲) and glass (■) surfaces.
cephalosporin group, 97% for aminoglycosides, 75% for quinolones, 66.6% for tetracycline and oxytetracycline, 33.3% for imipenem and 50% to the other antibiotics tested.

**Determination of MIC**

MICs of 14 antibiotics from different groups were tested against six selected *A. baumannii* isolates. The antibiotics included β-lactam groups, tetracycline, carbapenems, quinolones and others. The majority of *A. baumannii* isolates tolerated concentrations exceeding 512 μg mL⁻¹ of antibiotics from all groups. However, *A. baumannii* A2 and A3 were sensitive to colistin, tetracycline and imipenem. It was observed that more than 85% of *A. baumannii* isolates were highly resistant to β-lactam antibiotics. *Acinetobacter baumannii* strains were resistant to antibiotic nitrofurantoin to a lesser extent against gatifloxacin compared with β-lactam antibiotics. Resistance to tetracycline was low as compared with oxytetracycline at the same group. More than 66% of *A. baumannii* isolates were resistant to oxytetracycline at concentrations of > 1024 μg mL⁻¹. All *A. baumannii* isolates were sensitive to colistin at concentration of 2 μg mL⁻¹.

**Bacterial adhesion to urinary catheters and the effect of colistin**

The number of bacteria that adhered cm⁻² of catheter surface varied from 2250 (3.35 log₁₀) to 4900 (3.69 log₁₀). Treatment of cultures with 0.5 × MIC (1 μg mL⁻¹) and 0.25 × MIC (0.5 μg mL⁻¹) of colistin antibiotic significantly reduced the adhesion ability of all isolates (Table 2). Under a similar set of conditions, cultures treated with 0.5 × MIC colistin concentration could reduce the biofilms more than cells treated with 0.25 × MIC.
Plasmid isolation and determination of molecular weight

Multiple plasmids were found in 28 urinary isolates of *Acinetobacter* spp. *Acinetobacter baumannii* (25 strains) and *A. lwoffii* (three strains) harbored single or multiple plasmids. The number of plasmids observed in all *Acinetobacter* isolates ranged from one to nine. Molecular weights of these plasmids were in a range from 1.7 to 56.12 kb.

Plasmid curing

Four curing agents individually and in combination with heat were used to cure the antibiotic-resistant markers present in the three *A. baumannii* isolates that showed maximum biofilm formation. Plasmids pUPI802 (Cit) and pUPI804–807 (Cit) were cured by plumbagin with curing efficiencies of 4.5% from *A. baumannii* A3 (Table 3). The MICs of all cured clones ranged between < 32 and 64 μg mL⁻¹, whereas the...
wild-type parent strain had MIC values of $>1024 \mu \text{g mL}^{-1}$. The physical loss of the plasmid from cured strains was indicative of the plasmid-borne nature of antibiotic resistance. On the other hand, five plasmids of \textit{A. baumannii} A3 were cured but no differences in biofilm formation were observed between wild-type and plasmid-cured strains. Such results have also been reported recently in the case of uropathogenic \textit{E. coli} (UPEC) that harbor the plasmid pUTI89. Curing of this plasmid (UPEC) did not affect the growth or biofilm formation capabilities (Cusumano et al., 2010).

\section*{Plasmid transfer from \textit{Acinetobacter} to \textit{E. coli} and \textit{A. baylyi}}

Intergeneric conjugal transfer of plasmids pUPI 803–5 (A', Cp', Nf') from \textit{A. baumannii} A3 to \textit{E. coli} HB 101 was observed. The frequency of transconjugants was $1.5 \times 10^{-7}$ per recipient cell and these transconjugant colonies produced biofilm. Plasmid pUPI 806 (Cs', Cp') were transferred from \textit{A. baumannii} A3 to \textit{A. baylyi} 7054 trpE and frequency of transformation was $2.9 \times 10^{5}$ transformants $\mu \text{g}^{-1}$ plasmid DNA. All gene transfers (by conjugation and transformation) were confirmed on the basis of plasmid profile (O’Sullivan & Klaenhammer, 1993). MICS of transformants and transconjugants were found to be > 8-fold higher than wild-type parent strains.

\section*{Discussion}

In recent decades, increasing involvement of \textit{Acinetobacter} infections in hospital and their multidrug resistance nature has been an important observation (Dhakephalkar & Choparde, 1994; Tognim et al., 2004). Bacterial CSH of \textit{Acinetobacter} strains is known to be associated with pathogenicity, bacterial adhesion and biofilm formation (Absolon, 1988). Accordingly, we have evaluated the hydrophobicity of the isolates by determining the affinity of cells to xylene (Jones et al., 1996). \textit{Acinetobacter baumannii} strains A2 and A3 showed the highest CSH values as compared with the other strains. Attachment and biofilm formation on glass by clinical isolates of \textit{A. baumannii} is the property that is most likely to be associated with the capacity of this pathogen to survive in hospital environments, medical devices, and subsequently causes infections in compromised patients. However, there are only a few brief reports regarding this (Vidal et al., 1997; Tomaras et al., 2003). A recent study has also shown the biofilm formation, gelatinase activity and hemagglutination in \textit{A. baumannii} strains in relation to pathogenesis (Cevahir et al., 2009). In the present study, these initial observations were extended further by showing that the tested \textit{A. baumannii} strains attach to and form biofilm on different surfaces such as glass, polycarbonate, polypropylene and urinary catheters. It is important to note that some of these substances are used widely in the fabrication of medical environments. There is a positive relationship between the degree of bacterial hydrophobicity and adhesion to the abiotic surfaces (Costa et al., 2006). We have also found that selected strains of \textit{A. baumannii} with high HI formed biofilm under static as well as dynamic conditions. This directly indicates the ability of these biofilm formers to persist successfully in medical settings where pathogenic bacteria are subjected to the shearing forces of a liquid stream, such as those present in urinary catheters, or may make it possible to persist on surfaces such as those of hospital furniture (Bergogne et al., 1993).

The factors such as temperature, pH and salt concentration of the medium affect the production of biofilm. In the present study, \textit{A. baumannii} isolates showed maximum biofilm formation at 30°C, pH 6.0–7.0 and with NaCl concentration of 5.0 g L$^{-1}$. Microbial adherence to 96-well microtiter plates was obtained at a maximum level after 60 h at 30°C, as also reported by other researchers (Pruthi et al., 2003). Biofilm formation at different temperatures and the production of extracellular materials surrounding the attached cells was found to be in accordance with the reports

\begin{table}[h]
\centering
\caption{Effect of antibiotic colistin on number of \textit{Acinetobacter} spp. (log$_{10}$) adhered to the catheter surfaces}
\begin{tabular}{lccc}
\hline
Strain & Control* & Culture (0.25 $\times$ MIC) & Culture (0.5 $\times$ MIC) \\
\hline
\textit{A. baumannii} A1 & 3.35 & 2.38 & 2.12 \\
\textit{A. baumannii} A2 & 3.68 & 2.71 & 2.46* \\
\textit{A. baumannii} A3 & 3.69 & 2.72 & 2.51* \\
\textit{A. baumannii} A4 & 3.54 & 3.08 & 2.43 \\
\textit{A. baumannii} A5 & 3.43 & 3.01 & 2.41 \\
\textit{A. baumannii} A6 & 3.28 & 2.25 & 2.02 \\
\hline
\end{tabular}
\begin{tablenotes}
\item *Control represents cell counts of \textit{Acinetobacter baumannii} strains (log$_{10}$) without antibiotic treatment.
\item *Significant differences (P < 0.05) with respect to control experiments.
\end{tablenotes}
\end{table}

\begin{table}[h]
\centering
\caption{Plasmid curing efficiency of four curing agents against biofilm-forming strain of \textit{Acinetobacter baumannii} A3}
\begin{tabular}{lcccc}
\hline
Curing agent at 42°C & Sub-MIC (\mu g mL$^{-1}$) & No. of clones checked & Markers checked & % Curing & Markers cured \\
\hline
Plumbagin & 16 & 200 & A, Cu, Ci, Cs, Cp, Nf & 4.5 & Ci \\
Ethidium bromide & 128 & 200 & A, Cu, Ci, Cs, Cp, Nf & 00 & Nil \\
Acriflavin & 8 & 200 & A, Cu, Ci, Cs, Cp, Nf & 00 & Nil \\
Acridine Orange & 64 & 200 & A, Cu, Ci, Cs, Cp, Nf & 00 & Nil \\
\hline
\end{tabular}
\begin{tablenotes}
\item A, Ampicillin; Nf, nitrofurantoin; Cs, cefoperazone; Ci, ceftriaxone; Cu, cefuroxime; and Cp, cephalxin.
\end{tablenotes}
\end{table}
mentioned earlier (Towner et al., 1991; Bergogne-Bérézin & Towner, 1993). Tested A. baumannii strains have the ability to attach and form biofilms on plastic as well as glass surfaces. The obligate aerobic character of this pathogen favored dense cell conglomeration at the air–liquid interface (Van Pelt et al., 1985). Light and fluorescence microscopy showed that the biofilm formation was greater on polycarbonate surfaces than on glass. Data obtained by SEM confirmed the presence of cell stacks on glass, polycarbonate and urinary catheters. The pellicle formation may be representing exopolysaccharide synthesis (Towner et al., 1991; Tomaras et al., 2003).

The production of lectins in clinical strains is the other important factor in adhesion and pathogenesis and many of these adhesion molecules are principally carbohydrate-containing proteins (Doyle & Slifkin, 1994; Syed et al., 1999). Bacterial adhesion to urinary catheters is a factor in the development of bacteruria and septicaemia (Garner et al., 1988; Paragioiudaki et al., 2004). We found the presence of lectins in all biofilm-forming strains of A. baumannii. Independent of the clinical situation, the catheterized patient is often medicated with antibiotics. We also evaluated the in vitro adhesion ability of A. baumannii to catheter surfaces after treatment with sub-MIC doses of colistin, as these concentrations are incapable of killing bacteria, but can affect properties associated with bacterial virulence (Hostacka, 1999; Pompilio et al., 2010). We observed a reduction in bacterial adherence to catheter surfaces with sub-MIC concentration of colistin.

The presence of plasmids in A. baumannii is known to be associated with antibiotic resistance. This also enhances the ability of these isolates to transfer resistance markers to the other clinical strains in mixed infections by transformation or conjugation (Chopade et al., 1985; Patwardhan et al., 2008). The importance of studying gene transfer in natural environments has recently been emphasized by the emergence of multidrug-resistant bacteria (Davies, 1994). Because most bacteria in natural settings reside within biofilms, it follows that conjugation and probably transformation are likely mechanisms by which bacteria transfer genes within or between populations (Davey & O’Toole, 2000). The removal of biofilms made up of two or more bacterial communities is thus critical to decrease the incidences of gene transfer between bacteria. This may significantly decrease the formation of new multiple antibiotic-resistant strains (Johnson et al., 2006).

Based on the present study, we show the ability of A. baumannii isolates obtained from UTI to adhere to different abiotic surfaces under experimental conditions. The role of plasmids with antibiotic-resistant characteristics in gene transfer and resistance towards antibiotics in biofilm-forming strains has been established. Finally, biofilm formation as well as the potential ability of spreading the antibiotic-resistant markers to other pathogens has been highlighted.

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