Attachment to and biofilm formation on abiotic surfaces by *Acinetobacter baumannii*: involvement of a novel chaperone-usher pili assembly system

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*Acinetobacter baumannii* causes severe infections in compromised patients, survives on abiotic surfaces in hospital environments and colonizes different medical devices. In this study the analysis of the processes involved in surface attachment and biofilm formation by the prototype strain 19606 was initiated. This strain attaches to and forms biofilm structures on plastic and glass surfaces, particularly at the liquid–air interface of cultures incubated stagnantly. The cell aggregates, which contain cell stacks separated by water channels, formed under different culture conditions and were significantly enhanced under iron limitation. Electron and fluorescence microscopy showed that pili and exopolysaccharides are part of the cell aggregates formed by this strain. Electron microscopy of two insertion derivatives deficient in attachment and biofilm formation revealed the disappearance of pili-like structures and DNA sequencing analysis showed that the transposon insertions interrupted genes with the highest similarity to hypothetical genes found in *Pseudomonas aeruginosa*, *Pseudomonas putida* and *Vibrio parahaemolyticus*. Although the products of these genes, which have been named *csuC* and *csuE*, have no known functions, they are located within a polycistronic operon that includes four other genes, two of which encode proteins related to chaperones and ushers involved in pili assembly in other bacteria. Introduction of a copy of the *csuE* parental gene restored the adherence phenotype and the presence of pili on the cell surface of the *csuE* mutant, but not that of the *csuC* derivative. These results demonstrate that the expression of a chaperone-usher secretion system, some of whose components appear to be acquired from unrelated sources, is required for pili formation and the concomitant attachment to plastic surfaces and the ensuing formation of biofilms by *A. baumannii* cells.

INTRODUCTION

Members of the genus *Acinetobacter* are non-motile, ubiquitous Gram-negative bacteria that can be recovered from a wide range of sources such as soil, water, food products and medical environments (Bergogne-Berenzin & Towner, 1996). The latter source is of particular significance because *A. baumannii* is the *Acinetobacter* genomic species of greatest importance in human medicine. A large number of reports describe outbreaks of nosocomial infections that include urinary tract infections, secondary meningitis, wound and burn infections, and particularly nosocomial pneumonia (Bergogne-Berenzin et al., 1993, 1996). Some of the challenges in the prevention and treatment of the infections caused by this opportunistic pathogen are its remarkable widespread resistance to different antibiotics and its ability to persist in nosocomial environments and medical devices (Bergogne-Berenzin & Towner, 1996). *A. baumannii* survives for several days on inanimate objects and surfaces found normally in medical environments, even in dry conditions on dust particles. These survival properties most likely play a significant role in the outbreaks caused by this pathogen.

The potential ability of *A. baumannii* to form biofilms could explain its outstanding antibiotic resistance and survival properties. This possibility is supported by a very limited number of publications which showed that a clinical isolate of this bacterium is able to attach to and form biofilm...
structures on glass surfaces (Vidal et al., 1996, 1997). Bacterial biofilms, arrangements in which the cells are morphologically, metabolically and physiologically different from their planktonic counterparts (Stoodley et al., 2002), have been found on the surface of medical devices such as intubation tubes, catheters, artificial heart valves, water lines and cleaning instruments (Donlan & Costerton, 2002). The surfaces of all of these medical and dental devices are normal targets for colonization by complex microbial communities. Previous research efforts have shown that biofilm formation proceeds via a series of steps which, upon completion, produce a mature, three-dimensional structure on both biotic and abiotic surfaces. Some of the current working models for biofilm formation implicate the participation of either bacterial surface motility mediated by pili and flagella (O’Toole & Kolter, 1998a), the flagellum-mediated recruitment of planktonic cells by the developing biofilm from the liquid medium (Tolker-Nielsen et al., 2000) or the formation and growth of microcolonies formed as a consequence of the multiplication of cells attached to solid surfaces (Heydorn et al., 2000). While forming and establishing these multicellular structures, the cells composing them secrete exopolysaccharides which serve to fortify and maintain the structure of the biofilm. It is not well understood whether these steps and cell components are involved in the apparent ability of A. baumannii to form biofilms on abiotic surfaces. Furthermore, the mechanism by which this bacterium forms biofilms may pose a challenge because of its well-established non-motile phenotype (Bergogne-Berenzin & Towner, 1996).

In this work, we report the initial characterization of the biofilm structures formed by the A. baumannii 19606 prototype strain under different culture conditions. In addition, we have initiated the genetic and molecular analysis of some of the factors that are involved in this important cellular process that could explain the resistance and survival of this opportunistic pathogen under harsh conditions such as those found in patients and nosocomial environments.

**METHODS**

**Bacterial strains, plasmids and culture conditions.** The bacterial strains and plasmids used in this work are listed in Table 1. Luria–Bertani (LB) broth and agar (Sambrook et al., 1989) were used to maintain all bacterial strains. M9 minimal medium (Miller, 1972) was used for sliding agar motility assays and growth under chemically defined conditions. The effect of iron on biofilm formation was tested by using M9 minimal medium supplemented with 200 µM FeCl3, 200 µM ethylenediamine-di-(r-hydroxyphenyl) acetic acid (EDDHA) or 200 µM 2,2’-dipyridyl (DIP) to simulate iron-replete and two iron-limited conditions, respectively. The effect of different carbon sources on biofilm production was examined through the addition of either 10 mM sodium citrate, 25 mM sodium succinate, 50 mM ethanol, 0.5 % pyruvate, 0.5 % acetate or 0.5 % lactate to Simmons salts. The latter consisted of 0.2 g MgSO4 l−1, 1 g NH4H2PO4 l−1, 1 g K2HPO4 l−1 and 5 g NaCl l−1, adjusted to pH 7.0. Transmission electron microscopy (TEM) experiments were done using cells lifted from agar plates incubated at 37 °C. Scanning electron microscopy (SEM) and light microscopy experiments were done using cells cultured in LB broth. Swimming, swarming, sliding and twitching agar plates (Rashid & Kornberg, 2000) were used to test different types of cell motility. Plates were stab-inoculated through to the bottom with a sterile toothpick and incubated at 37 °C for 16 h.

<table>
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<tr>
<th>Strain/plasmid</th>
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<td>Hunger et al. (1990)</td>
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<td>pWH1266 harbouring csuE, Ap'</td>
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<td>Plasmid rescued by self-ligation of NdeI-digested DNA from mutant #144</td>
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<td>pMU349</td>
<td>Plasmid rescued by self-ligation of EcoRI-digested DNA from mutant #144</td>
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*Ap', ampicillin resistance; Km', kanamycin resistance; Tc', tetracycline resistance.*
General DNA procedures. Total DNA was isolated either by ultra-centrifugation in CsCl density gradients (Meade et al., 1982) or using a mini-scale method adapted from previously published research (Barcak et al., 1991). Plasmid DNA was isolated using commercial kits (Qiagen). DNA digestions were performed with restriction enzymes as indicated by the supplier (New England Biolabs) and size-fractionated by agarose gel electrophoresis (Sambrook et al., 1989). Southern blot analyses were conducted using standard protocols (Sambrook et al., 1989) using high-stringency conditions (Graber et al., 1998). The pUC4K aph gene, which encodes kanamycin resistance and was used as a probe to detect the insertion of the EZ::TN <R6K/ori/KAN-2> transposon, was PCR-amplified with Pfu DNA polymerase (Stratagene) and the primers 133 (5'-GGCCGATATATGCTGGGCCCTCCTG-3') and 134 (5'-AGGCCATATTCAACGGG-3'). The amplicon was purified using the GeneClean II kit (QiBiogene) and labelled with [α-32P]-dCTP (Feinberg & Vogelstein, 1983). The radioactive bands were detected with a Storm 860 scanner (Molecular Dynamics).

Transcriptional analysis of gene expression. Expression of polycistronic genes was tested by RT-PCR analysis using total RNA isolated from bacteria grown in LB broth as described by Wu & Jansen (1996). The RNA samples were treated with RNase-free DNase I (Roche) and used with an RT-PCR commercial kit (Qiagen), under the conditions suggested by the manufacturer. The amplicons were analysed by agarose gel electrophoresis (Sambrook et al., 1989). PCR of total RNA without reverse transcription was used to test for DNA contamination of RNA samples. The nature of the amplicons was confirmed by automated DNA sequencing.

Biofilm assays. One millilitre of fresh medium in borosilicate (15 × 125 mm), polystyrene (12 × 75 mm) or polycarbonate (12 × 75 mm) sterile tubes was inoculated with 0-01 ml of an overnight culture. Duplicate cultures for each sample were incubated for 8 h at 37°C. One tube was sonicated immediately for 5 s with a thin probe and the OD600 of the culture was determined to estimate total cell biomass. Supernatants of the other tube were aspirated and rinsed thoroughly with distilled water. The cells adhered to the tube wall were viewed using a Nikon Eclipse E400 microscope expressing the green fluorescent protein (GFP) were visualized by epifluorescence microscopy using a Nikon Eclipse E400 microscope (Nikon) with the same equipment was used to visualize samples stained with calcofluor white as described by Neu et al. (1999). A. baumannii 19606 cells harbouring pMU125 and expressing the green fluorescent protein (GFP) were visualized by epifluorescence microscopy using a Nikon Eclipse E400 microscope equipped with a SPOT digital camera (Diagnostic Instruments). The same equipment was used to visualize samples stained with calcofluor white as described by Neu et al. (2002). Colonies grown overnight on Tris-M9 agar were sampled with Formvar-coated gold grids, negative-stained with 1% sodium cacodylate and incubated at room temperature for 2 h. The fixative was removed and replaced immediately with distilled water to prevent sample dehydration. Small samples (1 × 1 cm) were cut from the sides of each plate, rinsed with distilled water, dehydrated with increasing concentrations of ethanol, ranging from 25 to 100%, and CO2 critical point dried. Samples were gold-coated and visualized with a JEOL T200 scanning electron microscope.

Transposition mutagenesis and rescue of interrupted genes. A. baumannii 19606 was mutagenized using the EZ::TN <R6K/ori/KAN-2> Tnp Transposome transposition mutagenesis system and electroporation as described by Dorsey et al. (2002). Transformants that grew after plating on LB agar containing 40 μg kanamycin ml⁻¹ were toothpicked into separate wells of 96-well polystyrene microtitre plates, each containing 200 μl LB broth. Plates were incubated at 37°C for 24 h, the culture supernatant was aspirated and the wells were rinsed with water and stained with crystal violet. Potential biofilm mutants were validated using tube and Petri dish assays as described above. The genomic regions harbouring the insertion of the EZ::TN <R6K/ori/KAN-2> transposon were rescued by self-ligation of EcoRI- or Ndel-digested DNA and electroporation into Escherichia coli EC100D pir cells. Plasmid DNA isolated from colonies that grew on LB agar containing 40 μg kanamycin ml⁻¹ was used as a template to determine the nucleotide sequence of the genomic DNA flanking the transposon element with the primers complementary to this insertion element that were supplied with the mutagenesis kit. Further extension of nucleotide sequences was done using custom-designed primers. DNA sequencing was conducted using the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Pharmacia Biotech) and an ABI 3100 automated DNA sequencer (Applied Biosystems). Sequences were examined and assembled using Sequencher 4.1.2 (Gene Codes). Nucleotide and amino acid sequences were analysed with DNASTAR, BLAST and the analysis tools available through the ExPASy Molecular Biology Server (http://www.expasy.ch), such as SIGNALP, PSORT, HMMTOP and TMHMM to predict the presence of signal peptides and the cellular locations of proteins. G+C content was determined with Artemis (http://www.sanger.ac.uk), using a 120 nt window.

Genetic complementation of a biofilm mutant. The A. baumannii 19606 csuE-like gene, whose disruption by the insertion of EZ::TN <R6K/ori/KAN-2> caused a biofilm-deficient phenotype in the derivative #144, was PCR-amplified from the parental strain genome with Pfu DNA polymerase and the primers 1671 (5'-C-GGATCCCTATGCTGGCTAAACATGGGCTGGGTTGTTGTG-3') and 1672 (5'-CCGAGATATATGCTGGCTAAACATGGGCTGGGTTGTTGTG-3') that included BamHI restriction sites. The blunt-ended amplicon was ligated into pCR-Blunt II-TOPO and transformed into E. coli Top10. Plasmid DNA, which was isolated from a kanamycin-resistant colony and proved to have an insert with the same nucleotide sequence as the parental DNA, was digested with BamHI, ligated into the cognate site of the shuttle vector pWH1266 and transformed into E. coli DH5α. Plasmid DNA was isolated from a colony that was resistant to 100 μg ampicillin ml⁻¹ and sensitive to 20 μg tetracycline ml⁻¹ and electroporated into A. baumannii 19606 #37 and #144 cells. Transformants that grew after overnight incubation at 37°C on LB agar containing 100 μg ampicillin ml⁻¹ were tested for their ability to form biofilms on plastic surfaces as described above. The presence of the complementing plasmid was verified by Southern blot analysis using csuE amplified from the parental strain as a probe.

RESULTS

Attachment to and formation of biofilms on abiotic surfaces

The initial assays showed that A. baumannii 19606 cells which were taken from an agar plate with a bacteriological
loop, deposited on the surface of a Petri dish or a Teflon strip and left for 10–15 min at room temperature remained tightly adhered after washing with tap water and staining with crystal violet. Incubation of A. baumannii 19606 cells overnight in LB broth without shaking at 37 °C either in polystyrene tubes or square Petri dishes also resulted in their adherence to these surfaces. In contrast, no adherence was detected with E. coli DH5α cells cultured under similar conditions (compare tubes and plates shown in Fig. 1a and b). These observations were confirmed further when cell attachment to plastic was quantified by relating the total cell mass to the stain retained by the attached cells as described in Methods. It is interesting to note that the A. baumannii 19606 cells form denser aggregates at the liquid–air interface than on the side and bottom surfaces of the tubes and plates. Furthermore, the biofilm grows upwards from the liquid–air interface onto the walls of the plate (Fig. 1b), a phenomenon that is not caused by the movement of the broth, since the inoculated plate was incubated without any movement until stained with crystal violet.

Quantitative analysis showed that while the amount of A. baumannii 19606 cells attached to polystyrene and polypropylene is similar, their attachment to borosilicate is significantly reduced when tested under the same experimental conditions (Fig. 2a). Culture shaking also reduced the amount of cells attached to these three abiotic surfaces when compared with cultures incubated stagnantly (Fig. 2a). The values shown in Fig. 2(a), particularly those obtained with glass tubes, do not represent background values since crystal violet does not attach by itself to the surface of borosilicate. A. baumannii 19606 attaches to polystyrene tubes when cultured stagnantly at different temperatures, although this process is more efficient at 30 °C than at 37 °C (Fig. 2b). The addition of inorganic iron to M9 minimal medium reduced the amount of attached cells, while the supplementation of this chemically defined medium with the iron chelators EDDHA or DIP increased cell attachment significantly (Fig. 2c). In contrast, no significant effects were observed when the cells were cultured in a chemically defined medium supplemented with different carbon sources such as citrate, succinate, ethanol, pyruvate, acetate or lactate (data not shown).

**Microscopy analysis of cells attached to plastic surfaces**

Light microscopy examination of A. baumannii 19606 cells attached to the bottom surface of a Petri dish and stained with crystal violet showed the presence of discrete cell clumps (Fig. 3a), which did not coalesce to form at least a monolayer even after several days of incubation with or without renewing the culture medium. In contrast, compact cell aggregates were seen on the sides of the plates at the liquid–air interface. Similar results were obtained when A. baumannii 19606 cells producing GFP encoded by the recombinant plasmid pMU125 were examined with epi-fluorescence microscopy (Fig. 3b). This figure also shows that the fluorescent cellular aggregates are interspersed with areas devoid of cells, an arrangement that is very similar to those described in other bacteria capable of forming biofilms on abiotic surfaces, such as *Pseudomonas aeruginosa* (Lawrence et al., 1991). Fluorescence microscopy of samples stained with calcifluor white showed that the cells attached either to the walls or the bottom of the plates were embedded within a blue fluorescent material (Fig. 3c). This non-specific UV-excitable dye that binds to exopolysaccharides such as those produced by *Rhizobium meliloti*.
(Leigh et al., 1985) was recently used to study biofilms formed by Salmonella enteritidis (Solano et al., 2002). These reports together with our data support the hypothesis that A. baumannii produces exopolysaccharides that are part of the cellular structures formed by this bacterium when it grows attached to a plastic surface.

SEM analysis of samples similar to those used for light microscopy showed that while few cells were clustered together on the sides of the plate below the meniscus of the liquid medium, compact cell conglomerates separated by channel-like spaces were formed at the meniscus of the broth (compare Fig. 4b and c). Much denser and tighter conglomerates were formed by cells located just above the meniscus, which appear to form multilayer structures, with the top cell layer covered with a film that most likely represents the exopolysaccharides produced by this bacterium (Fig. 4a). Alternatively, this film could also have been the result of the dehydration of the cell aggregates and the exopolysaccharides that surround them, which are located immediately above the liquid-air interface, during incubation. SEM also showed that the cells were linked to each other through extracellular appendages that resemble pili structures (see insets of Fig. 4a and b). The presence of such a type of appendage was investigated by TEM of cells cultured on agar plates and stained with ammonium molybdate. This approach showed the presence of structures that resemble pili projecting from the surface of the cells (Fig. 5a), which were found to be fairly equally distributed on the cell surfaces without any particular organization.

Cell motility and the presence of cellular appendages have been implicated in the formation of biofilms by other bacteria (O'Toole & Kolter, 1998a; Tolker-Nielsen et al., 2000). However, members of the Acinetobacter genus, including A. baumannii, are taxonomically defined to lack flagella and thus are non-motile (Bergogne-Berenzin & Towner, 1996). To confirm this inability to move, we tested 19606 cells by inoculating defined solid media used to detect swimming, swarming, sliding and twitching motility as described by Rashid & Kornberg (2000). These assays proved that A. baumannii 19606 lacked the ability to move.
via these mechanisms under the experimental conditions used in this study.

**Genetic analysis of cell attachment and biofilm formation**

The EZ::TN \(<\text{R6K}_{\text{ori}}/\text{KAN-2}\>\) Tnp Transposome system from Epicentre was used to initiate the identification and characterization of some of the genetic determinants required by *A. baumannii* 19606 to attach to and form biofilms on plastic surfaces. As we reported recently (Dorsey *et al.*, 2002), screening of insertion derivatives of this strain resulted in the identification of mutants affected in cell attachment and biofilm formation without affecting their growth in LB broth. The rescue cloning and initial nucleotide sequence analysis of the mutant #144 resulted in the identification of a gene encoding a protein highly similar to that encoded by the *Vibrio parahaemolyticus csuE* gene. TEM analysis of mutant #144 proved that the disruption of the *A. baumannii* 19606 csuE-like gene resulted in the disappearance of the pili detected in the parental strain (compare Fig. 5a and b). Although not shown, SEM analysis of the side of the plates in which the mutant #144 derivative was incubated showed the presence of very few cell clusters, with no more than two or three cells grouped together that did not display pili on their surfaces (data not shown).

The role of the *A. baumannii* 19606 csuE-like gene was confirmed further by genetic complementation of the insertion derivative with the parental gene PCR-amplified and cloned in the shuttle vector pWH1266 (Hunger *et al.*, 1990). Electroporation of the recombinant plasmid pMU243 into the *A. baumannii* 19606 #144 insertion derivative restored its ability to attach and form biofilms in a fashion similar to that displayed by the 19606 parental strain (sample 2 in Fig. 1a). In contrast, the electroporation of pWH1266 did not change the phenotype of the biofilm-deficient derivative, which produced an image identical to that displayed by sample 1 in Fig. 1(a). SEM and TEM analyses also showed that the introduction of the parental csuE-like gene restored the ability of *A. baumannii* 19606 #144 to form structures similar to those formed by the parental strain (Fig. 4d–f) and the concomitant presence of

![Fig. 4. SEM of *A. baumannii* 19606 cells attached to square Petri dishes. Attachment of the parental strain (left) and the insertion derivative #144 harbouring plasmid pMU243 (right) above (a and d), at (b and e) and below (c and f) the liquid–air interface. Bars: (a, c, d–f) 5 μm; (b) 10 μm; all insets, 1 μm.](image-url)
pili on the surface of the mutant cells (Fig. 5c). Southern blot analysis of total DNA isolated from A. baumannii 19606 #144 and the complemented mutant probed with the csuE-like gene validated the attachment and biofilm results by confirming the presence of the complementing plasmid pMU243 as an independent replicon without detectable rearrangements (data not shown).

Sequence analysis of plasmid DNA rescued from mutant #144

Sequence analysis of plasmids pMU348 and pMU349, which were rescued by self-ligation of NdeI- and EcoRI-digested total DNA isolated from this biofilm mutant, showed that the A. baumannii 19606 csuE-like gene is the last component of a gene cluster that encompasses six ORFs (Fig. 6a). The mean G+C content of this region is 37.5 mol%; however, ORF 2 (28.5 mol%) and 3 (29.7 mol%) displayed a significantly lower value than the mean (Fig. 6b and Table 2). This observation suggests that these two ORFs were acquired from a source different from that of the remaining ORFs that compose this apparently polycistronic locus, whose G+C content is closer to the 40–43 mol% values assigned to different A. baumannii isolates (Bouvet & Grimont, 1986). The polycistronic nature of this locus was confirmed by RT-PCR analysis, which produced amplicons with the predicted sizes (Fig. 7), and nucleotide sequences when the appropriate pairs of primers were used. None of these amplicons could be detected when total RNA was used as a template for PCR.

The first ORF encodes a potential protein with a predicted 25 aa signal peptide, which showed the highest similarity to the P. aeruginosa hypothetical protein PA4648 (Stover et al., 2000) and the Pseudomonas putida PP2358–PP2360 hypothetical proteins (Nelson et al., 2002) (Table 2). All these proteins contain the conserved domain COG5430, which has been identified in other hypothetical proteins produced by bacteria such as P. aeruginosa, Ralstonia solanacearum and Yersinia pestis. These proteins were annotated as potential secreted proteins related to the type 1 pili subunit CsUA and CsUB proteins. Significant homology was also detected with the Myxococcus xanthus spore protein U, which is produced as a secretory precursor and then secreted across the membrane to be assembled on the spore surface (Gollop et al., 1990). Interestingly, the protein U and other proteins annotated as protein U-like proteins also showed significant similarity to the conserved domain COG5430. This similarity may reflect the fact that most of the proteins that belong to this family have been annotated as secreted proteins and therefore may use similar secretion mechanisms. The product of ORF 2, which is predicted to have a 31 aa signal peptide, displayed significant similarity only to the hypothetical V. parahaemolyticus CsUA protein (Table 2). The predicted product of ORF 3 is a protein with a potential 20 aa signal sequence that is related to the proteins PP2360, PA4648 and CsUB from P. putida, P. aeruginosa and V. parahaemolyticus, respectively (Table 2). Interestingly, all these proteins contain a sequence highly related to the conserved domain COG5430 that has been found in proteins annotated as type 1 pili subunit CsUA/B or CsUB in different Gram-negative bacteria, as well as sequences related to the spore protein U. Further analysis using GAP showed that the identity of the A. baumannii 19606 CsUA/B, CsUA and CsUB proteins ranged from 23.7 to 27.7%, while their similarity was between 34.1 and 36.6%.

The predicted product of the fourth ORF is highly related to the hypothetical PP2361, CsUC and PA4651 proteins of P. putida, V. parahaemolyticus and P. aeruginosa, respectively.

**Fig. 5.** TEM of A. baumannii 19606 cells cultured on agar and stained with ammonium molybdate. (a) 19606 parental strain. (b) Insertion mutant #144. (c) Insertion mutant #144 harbouring the plasmid pMU243. Bars, 100 nm.
(Table 2). Analysis with BLASTP showed that all four proteins are highly related to fimbrial chaperones and contain the COG 3121.1 and Pfam 00345.6 conserved domains, which have been either described or annotated as pili assembly factors, with the FimC and PapD chaperones being some of the best characterized members of this protein family that

**Table 2.** Sequence analysis of the ORFs located within the *csu* locus of *A. baumannii* 19606

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<th>G+C (mol%)</th>
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<td>39.7</td>
<td>832/92-7</td>
<td><em>P. putida</em> PP2362</td>
<td>e-144</td>
<td>38.0/46-0</td>
<td>AAN67975</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>P. aeruginosa</em> PA4652</td>
<td>e-143</td>
<td>41.7/50-4</td>
<td>Q9HVE0</td>
</tr>
<tr>
<td>6</td>
<td>42.5</td>
<td>339/36-6</td>
<td><em>P. putida</em> PP2363</td>
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<td>34.2/38-9</td>
<td>AAN67976</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>V. parahaemolyticus</em> CsuE</td>
<td>2e-23</td>
<td>33.2/41-6</td>
<td>AF339087</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td><em>P. aeruginosa</em> PA4653</td>
<td>6e-18</td>
<td>29.8/38-1</td>
<td>Q9HVD9</td>
</tr>
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</table>

*Identification of bacterial products is based on BLASTP analysis.
†The E values of the highest and more significant matches obtained during the BLASTP analyses are shown.
‡Values were obtained with the GCG GAP program.
are involved in the biogenesis of pili in *E. coli* (Hermanns *et al.*, 2000; Sauer *et al.*, 2000). The expression and role of this gene is supported by the phenotype of the derivative #37, which harbours a transposon insertion in *csuC* (Fig. 6a) and displays the same biofilm-deficient phenotype as mutant #144 (data not shown). TEM analysis showed that CsuC-deficient cells do not have pili on the cell surface, an observation that confirms the role of this protein in the assembly of these cell appendages (data not shown). The product of ORF 5 is a large protein that is highly similar to the hypothetical proteins PP2363 and PA4652 of *P. putida* and *P. aeruginosa*, respectively (Table 2). These three proteins contain sequences related to COG3188 and Pfam 00577, conserved domains that have been described in fimbrial usher proteins involved in the biogenesis of Gram-negative bacterial pili. This protein family includes the FimD and PapC usher proteins that are required for the biogenesis of pili (Klemm *et al.*, 1985; Thanassi *et al.*, 1998).

The last ORF of this *A. baumannii* 19606 gene cluster, whose disruption by the insertion of the EZ::TN <R6K/gyrI/KAN-2> transposon (Fig. 6a) resulted in the isolation of the attachment and biofilm deficient derivative #144, encodes a predicted protein that has a 27 aa signal peptide. BLASTP analysis showed that the most related proteins are all hypothetical proteins, with the highest similarity to the PP2363, CsuE and PA4653 proteins of *P. putida*, *V. parahaemolyticus* and *P. aeruginosa*, respectively (Table 2). This predicted *A. baumannii* protein has sequences highly related to the COG5430 conserved domain that has been found in uncharacterized secreted proteins in *P. aeruginosa*, *Y. pestis*, *Ralstonia solanacearum* and *Rickettsia conorii*. Furthermore, a search of the Pfam database with MOTIFS CAN showed that the product of this ORF has a motif with some similarity to fimbrial proteins found in different bacteria. As described for the products of ORFs 1 and 3, the product of ORF 6 also has similarity with proteins harbouring the protein U domain found in the family of spore coat proteins. Fig. 6(c) shows that the *A. baumannii* 19606 gene cluster has the same number of genes and genetic organization as in *P. aeruginosa* (Stover *et al.*, 2000) and *Y. pestis* (Parkhill *et al.*, 2001), while the cognate loci in *P. putida* (Nelson *et al.*, 2002) and *V. parahaemolyticus* (GenBank accession no. AF339087; Makino *et al.*, 2003) are one ORF longer and shorter, respectively.

It is worthy to note that all the genes and gene products described in Table 2 are hypothetical bacterial elements described during the annotation of the cognate genomes. Therefore, neither their expression nor biological role(s) were tested experimentally as we have done with the *A. baumannii* 19606 *csu* locus. Furthermore, the *P. aeruginosa* PA4648–PA4653 gene cluster, which showed significant similarity to the *A. baumannii* 19606 *csu* locus, is different from the *cupA1–A5* fimbrial gene cluster that specifies a chaperone-usher pathway that was found to be involved in the formation of biofilm formation by this pathogen (Vallet *et al.*, 2001). Nevertheless, the *A. baumannii* 19606 *csu* locus showed significant similarity with the *cupA* cluster when compared with BLASTX. However, the similarity was only with CupA3 (E value 1e-20), the usher component of this *P. aeruginosa* secretory system that was annotated as the PA2130 protein. Therefore, the expression and biological role of the *P. aeruginosa* PA4648–PA4653 gene cluster remains to be tested.

**DISCUSSION**

Several reports have shown that *Acinetobacter* environmental isolates form biofilm communities composed of different bacterial species. In contrast, only two brief reports describe the ability of *A. baumannii* clinical isolates to attach to and form biofilms on glass surfaces (Vidal *et al.*, 1996, 1997), a property that is most likely to be associated with the capacity of this pathogen to survive in hospital environments and medical devices, and cause severe infections in compromised patients (Bergogne-Berenzin & Towner, 1996). In this report we have extended these initial observations by showing that the prototype strain 19606 also attaches to and forms biofilms on the surface of different plastics (polystyrene, polypropylene and Teflon), some of which are used in the fabrication of medical devices. It is also evident that this bacterium forms biofilms under static as well as dynamic conditions, although the latter results in less biofilm when compared with samples incubated stagnantly. This property could explain the ability of this pathogen to persist successfully in medical environments where cells could be subjected to the shearing forces of a liquid stream, such as those found with catheters and respiratory tubes, or allowed to persist on less disturbed surfaces such as those of hospital furniture and bed linen (Bergogne-Berenzin & Towner, 1996). The formation of biofilms at different temperatures and the presence of extracellular material surrounding the attached cells that is stained with calcofluor white are also in accordance with the ability of this pathogen to grow within a wide range of temperatures and produce capsule and exopolysaccharides (Bergogne-Berenzin & Towner, 1996; Towner *et al.*, 1991). It is also clear that *A. baumannii* 19606 attaches to and forms biofilm structures on hydrophobic (plastic) as well as hydrophilic (glass) surfaces, with
much denser cell conglomerates located at the liquid–air interface. This observation is significant because *A. baumannii* is a strict aerobic bacterium, a phenotype that could explain this behaviour as well as the observation that the bottom of the plates or tubes did not have a contiguous cell layer even after extended incubation. No significant effects on biofilm formation were observed when the cells were cultured in a chemically defined medium supplemented with different carbon sources. In contrast, the incubation of *A. baumannii* 19606 cells under iron limitation resulted in a significant increase of biofilm when compared to that obtained with cells cultured under iron-rich conditions. This expressional behaviour could be explained by the presence of the nucleotide sequence 5′-TACAAATCTAAAATCATTATA-3′ 10 nt upstream of the first ORF, which matches 13 of the 19 nt of the binding site for the Fur iron repressor protein that controls the expression of iron-regulated protein in bacteria (Calderwood & Mekalanos, 1988). These observations are quite significant since these bacterial cells would most likely be exposed to iron-limiting conditions either in the host or the nosocomial environment (Neilands, 1981).

The light microscopy of crystal-violet-stained cells and fluorescence microscopy of GFP-expressing bacteria showed that the conglomerates located at the liquid–air interface indeed form biofilm structures similar to those described for other bacteria, with channels that would provide nutrients and remove waste products. The presence of these channels is supported further by the data obtained by SEM, which showed stacks of cells surrounded by open areas on the sides of the plates at the liquid–air interface (Fig. 4b). The cell stacks appear to be 4–5 cells tall with the cells attached to each other by pili-like structures and amorphous material, with the latter being more evident in the structures formed just above the meniscus of the broth. The pili-like structures, whose presence was confirmed by TEM analysis, were also seen on the surface of *A. calcoaceticus* RAG-1, a hydrocarbon-degrading environmental isolate that adheres to plastic surfaces and hexadecane droplets (Rosenberg et al., 1982). Interestingly, a mutation that abolished the formation of these cell-surface appendages impaired the ability of this bacterium to attach to hydrophobic surfaces and grow in the presence of hexadecane as sole carbon source. This attachment phenotype that depends on the formation of thin filaments is very similar to what we have observed with *A. baumannii* 19606, which is discussed in more detail below. The amorphous material seen between cells, cell stacks and particularly covering the top cell layer formed above the broth meniscus may represent exopolysaccharides, compounds known to be produced by environmental and clinical isolates of *Acinetobacter* (Towner et al., 1991). Taken together, the data indicate that environmental and clinical members of the *Acinetobacter* genus use similar strategies to attach to solid surfaces and to initiate the formation of biofilm structures that allow them to persist and proliferate under harsh conditions.

Cell motility mediated either by appendages such as flagella and pili or the action of glycopeptidolipids is required for biofilm formation by bacteria such as *P. aeruginosa* (O’Toole & Kolter, 1998a), *P. fluorescens* (O’Toole & Kolter, 1998b) and *Mycobacterium smegmatis* (Recht et al., 2000), respectively. The lack of this cell behaviour was one of the criteria used by Baumann et al. (1968) to name this genus *Acinetobacter*, although twitching and gliding motility have been observed when some isolates were tested on semi-solid media (Towner et al., 1991). All our attempts failed to detect any type of motility with the *A. baumannii* 19606 prototype strain, indicating that its ability to form biofilms does not depend on this cell property, at least under the experimental conditions used in this work. Based on this behaviour, it is possible to speculate that the multiplication and growth of cells and microcolonies already attached to solid surfaces is the mechanism by which this strain forms biofilms on plastics and glass, as described for some *Pseudomonas* strains (Heydorn et al., 2000).

The genetic approach used in this work proved that the presence of pili-like structures on the surface of *A. baumannii* 19606 cells is essential in the early steps of the process that leads to the formation of biofilm structures on plastic surfaces. The disruption of the *csuC* and *csuE* ORFs resulted in non-piliated cells and abolished cell attachment and biofilm formation. These defects were restored to levels similar to those of the parental strain when the insertion derivative #144 was electroporated with a shuttle vector harbouring the wild-type copy of the *csuE* gene. In contrast, this recombinant construct could not reverse the phenotype of the mutant #37, in which the insertion interrupted the *csuC* chaperone-encoding gene, to that of the parental strain. Taken together, the data show that this is indeed a polycistronic operon that encodes functions required for pili assembly and biofilm formation. Based on the nucleotide composition, it appears that part of this gene cluster has been acquired by *A. baumannii* 19606 from an unrelated source by lateral gene transfer, a possibility that is in accordance with the well-known natural competence and ability of members of this genus to acquire DNA easily from different sources (Towner et al., 1991).

While the translational products of the fourth and fifth ORFs are the most recognizable since they are highly related to chaperone and usher bacterial proteins, respectively, the four remaining ORFs encode hypothetical proteins potentially involved in pili assembly. Searches with PSI-BLAST, SCANPROSITE and MOTIFSCAN failed to identify the ORF encoding the major pilus protein among the four ORFs whose predicted products are not related to chaperone and usher proteins. On one hand, this observation may indicate that the *A. baumannii* 19606 gene encoding this subunit is one of the four ORFs located in this cluster, although its translation product is not significantly related to known pilus subunit sequences. On the other hand, it is possible that the gene encoding this subunit is located outside of this
gene cluster. This analysis also showed that the systems most related to that of *A. baumannii* 19606 are found in the human pathogens *P. aeruginosa* (Stover *et al.*, 2000) and *Y. pestis*, and the environmental bacterium *P. putida* (Nelson *et al.*, 2002), all of which have been annotated as hypothetical genes and proteins. In addition, a similar locus was found in the strains BB22 (GenBank accession no. AF339087) and RIMD 2210633 (KXXV237) (Makino *et al.*, 2003) of the food-borne pathogen *V. parahaemolyticus*. This observation suggests that the csu operon is widespread among unrelated bacteria, which could play a central role in the ability of these micro-organisms to attach to and form biofilms on abiotic surfaces, a property that would allow them to persist in their natural environments.

In summary, the results presented in this study demonstrate the ability of the prototype strain 19606 of the opportunistic pathogen *A. baumannii* to attach to and form biofilms on abiotic surfaces under different experimental conditions. In addition, the genetic approach used in this work proved that the expression of csuC and csuE, which belong to a gene cluster related to bacterial loci encoding secretion and pili assembly functions and the production of pili are required in the early steps of the process that leads to biofilm formation. Prior to this study, the loci most closely related to the *A. baumannii* 19606 csu locus were annotated as hypothetical genes and proteins. This study demonstrates the expression of and identifies a biological function for one member of this uncharacterized group of operons of the chaperone-usher superfamily.

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**REFERENCES**


