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**Author:** Breij, Anastasia de  
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Chapter 3

CsuA/BABCDE-dependent pili are not involved in the adherence of *Acinetobacter baumannii* ATCC19606<sup>T</sup> to human airway epithelial cells and their inflammatory response

Anna de Breij<sup>1</sup>, Jennifer Gaddy<sup>2</sup>, Joke van der Meer<sup>3</sup>, Roman Koning<sup>3</sup>, Abraham Koster<sup>3</sup>, Peterhans van den Broek<sup>1</sup>, Luis Actis<sup>2</sup>, Peter Nibbering<sup>1</sup>, Lenie Dijkshoorn<sup>1</sup>

<sup>1</sup>Dept. of Infectious Diseases, Leiden University Medical Center, Leiden, the Netherlands,
<sup>2</sup>Dept. of Microbiology, Miami University, Oxford, OH, USA, <sup>3</sup>Dept. of Molecular Cell Biology, Section Electron Microscopy, Leiden University Medical Center, Leiden, the Netherlands

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Abstract

Acinetobacter baumannii is a nosocomial pathogen responsible for outbreaks of infection worldwide. The factors associated with its ability to colonize/infect human hosts are largely unknown. Adherence to host cells is the first step in colonization/infection, which can be followed by biofilm formation. A. baumannii ATCC19606T biofilm formation on abiotic surfaces depends on expression of the CsuA/BABCDE chaperone–usher pili assembly system. The present study focused on the involvement of CsuA/BABCDE-dependent pili in the interactions between A. baumannii 19606T and human bronchial epithelial cells and sheep erythrocytes. Light microscopy analysis revealed that CsuE-mutant #144 adhered to more bronchial epithelial cells than the parental strain. Similar amounts of interleukin (IL)-6 and IL-8 were produced by bronchial epithelial cells in response to these two bacterial strains. Scanning electron microscopy revealed the presence of two types of surface extensions on ATCC19606T, i.e., short (29 nm; 5–140 nm) pili and long (260 nm; 143–1008 nm) extensions. The latter were not observed on the CsuE-mutant and therefore are likely the previously described CsuA/BABCDE-encoded extensions. We conclude that CsuA/BABCDE-dependent pili are not involved in adherence of A. baumannii ATCC19606T to bronchial epithelial cells. The structure of the short pili and their possible role in adherence to human cells requires further investigation.
Introduction

During the last decades, *Acinetobacter baumannii* has emerged globally as an important nosocomial pathogen that gives rise to outbreaks of colonization and infection of critically ill, hospitalized patients [3], [6], [12] and [16]. The strains involved are frequently multidrug-resistant (MDR) and have the ability to spread epidemically among patients and survive in the hospital environment [25]. The recent appearance of carbapenem resistance in these strains is a major source of concern [17].

Despite the widely documented problems regarding nosocomial *A. baumannii* infections, little is known about the mechanisms that contribute to the epidemicity and pathogenicity of this species. It is generally thought that the intimate interaction between bacteria and their host begins with the adherence of the microorganism to host tissues followed by colonization of the host [2]. During colonization, bacteria may form microcolonies and produce exopolysaccharides resulting in a highly structured microbial community called biofilm [5]. Bacteria within a biofilm exhibit increased resistance to antimicrobial compounds, including those elicited by the host’s immune response [5]. The progression from colonization to infection of the host depends on the balance between the host’s immune response and the virulence of the bacterium.

Several in vitro studies have shown that particular strains of *A. baumannii* can adhere to human cells [13] and form biofilms on abiotic surfaces [18], [22], [26] and [27]. Tomaras et al. have demonstrated that the ability of *A. baumannii* strain ATCC19606 to form pili, adhere to and form biofilms on abiotic surfaces depends on the expression of *csuE*, which is part of the CsuA/BABCDE chaperone–usher pili assembly system [22]. Pili may not only promote adherence and biofilm formation, but the coupling of pili to host cell receptors may also induce inflammation through the production of inflammatory mediators, including chemokines and cytokines [21].

The aim of the present study was to investigate the involvement of *A. baumannii* CsuA/BABCDE-mediated pili in adherence to vertebrate cells and the inflammatory response of human bronchial epithelial cells.

Materials and Methods

**Bacterial strains and culture conditions**

*A. baumannii* type strain ATCC19606\(^T\) and the *csuE* isogenic insertion mutant ATCC19606 #144 [22] were used. High-resolution fingerprinting by AFLP analysis confirmed that both isolates belong to the same strain (similarity of 99.4% as calculated by the Pearson
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Upon verification, CsuE-mutant #144 did not form a biofilm on polystyrene after 24 h incubation at 28°C and 37°C. Of note, biofilm formation on abiotic surfaces was tested as described before [22].

Bacteria were preserved for prolonged periods in nutrient broth supplemented with 20% (v/v) glycerol at −80°C. Prior to each experiment, inocula from frozen cultures were grown overnight at 30°C on sheep blood agar plates (BioMerieux, Boxtel, The Netherlands) or trypticase soy agar (BD, Sparks, MD, USA) containing 5% sheep erythrocytes. For experiments, subcultures were made under these conditions.

**Bronchial epithelial cell culture**

Human bronchial epithelial *H₂₉₂* cells (ATCC CRL-1848, Mansas, VA, USA) were cultured as described previously [13]. Briefly, *H₂₉₂* cells were cultured in RPMI-1640 medium supplemented with 2 mM L-glutamine, 50 μg/ml streptomycin, 1 mg/ml sodium penicillin G, and 10% heat-inactivated fetal calf serum (FCS) (all from Gibco, Invitrogen, Breda, The Netherlands), further referred to as culture medium, at 37°C/5% CO₂ in 25 cm² tissue culture flasks (Greiner Bio-One, Frickenhausen, Germany). At confluency, cells were trypsinized and 2x10⁵ *H₂₉₂* cells were resuspended in 0.5 ml of RPMI-1640 supplemented with 2 mM L-glutamine and 10% FCS, further referred to as RPMI medium, in 24-wells plates.

**Bacterial adherence to bronchial epithelial cells**

The adherence of bacteria to human bronchial epithelial cells was determined as described [13]. Briefly, 2x10⁵ *H₂₉₂* cells were cultured for 24 h in RPMI medium on 13-mm diameter plastic coverslips (Thermanox, Nunc, Rochester, NY, USA) placed in 24-well plates. Bacteria were cultured overnight at 30°C on blood agar plates and suspended in RPMI medium to an optical density at 600 nm of 0.6 (SmartSpec 3000, BioRad), corresponding to a concentration of ~1x10⁸ colony forming units (CFU)/ml, which was verified afterwards by standard vital counting. *H₂₉₂* cells were incubated for 1 h at 37°C with 1x10⁷ (range 7x10⁶–4x10⁷) bacteria. Next, each coverslip was removed and washed five times with prewarmed PBS. The cells on the coverslip were fixed with methanol and stained with Giemsa, after which the coverslips were mounted onto microscope slides. Bacterial adherence was quantitated by light microscopy examination. For each coverslip, a minimum of 800 *H₂₉₂* cells were inspected and the percentage of epithelial cells associated with at least one bacterium was determined. In addition, 100 *H₂₉₂* cells with attached bacteria were analyzed to assess the number of bacteria associated with human bronchial epithelial cells. Each bacterial strain was examined in duplicate in four independent experiments performed on different days.
Stimulation of bronchial epithelial cells

Cytokine production in bronchial epithelial cells in response to the presence of bacteria was determined using a stimulation assay. Bacteria were cultured overnight at 30°C in Luria–Bertani (LB) medium (10 g bactotryptone, 5 g yeast extract (both from BD, Sparks, MD, USA), 5 g sodium chloride (Merck, Darmstadt, Germany) in 1000 ml distilled water), washed twice with PBS and suspended in RPMI medium to a concentration of \(1 \times 10^8\) CFU/ml as calculated from the absorbance of a suspension at 600 nm. Approximately \(2 \times 10^5\) H292 cells were cultured in 0.5 ml of RPMI medium in 24-well plates. At 85–90% confluency, H292 cells were washed once with prewarmed PBS and incubated at 37°C/5% CO2 with \(1 \times 10^7\) bacteria. After 1 h, H292 cells were washed five times with prewarmed PBS to remove non-adherent bacteria and incubated again in fresh RPMI medium. After 23 h incubation at 37°C, supernatants were collected and stored at \(-20°C\), until determination of the levels of the inflammatory mediators interleukin (IL)-6 and IL-8 by ELISA. In each experiment, RPMI medium alone was added to the cells in order to obtain background values. Five independent experiments were performed in triplicate.

Determination of inflammatory mediators

The levels of IL-6 and IL-8 in culture supernatants were determined by enzyme-linked immunosorbent assays (ELISA, Biosource, Camarillo, CA, USA) according to the manufacturer’s instructions. The lower limit of detection was 15 pg/ml for IL-6 and 7 pg/ml for IL-8.

Scanning electron microscopy

For scanning electron microscopy (SEM), bacteria from an overnight culture on blood agar were suspended in PBS and subsequently fixed for 1 h at room temperature with 1.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (1:1). Fixed bacteria were washed twice in PBS and transferred to poly-l-lysine-coated glass slides. After 1 h incubation at room temperature, bacteria were fixed to the slides with 1.5% glutaraldehyde for 30 min at room temperature. Thereafter, slides were washed twice in PBS, dehydrated through a series of graded ethanol, critical-point-dried, and finally coated with a layer of palladium-gold. The bacteria were examined using either a JEOL JSM-6700F or a Zeiss Supra 35VP field emission scanning electron microscope.

Detection of CsuA/B protein

For detection of the CsuA/B protein, bacteria from an overnight culture on blood agar were used to prepare whole-cell lysates as described before [7]. Proteins were size-fractionated on 12.5% polyacrylamide gels [1], transferred to nitrocellulose [24], and
incubated with polyclonal anti-CsuA/B antiserum raised in rabbits using recombinant CsuA/B (overexpressed and purified by Ni-affinity column chromatography) as antigen [23]. The immunocomplexes were detected by chemiluminescence using horse radish peroxidase (HRP)-labeled protein A. Protein concentrations were determined as described previously [4].

**Detection of acuA gene**
The presence of the acuA gene, a pilus-encoding gene identified in another Acinetobacter species, *A. baylyi*, was determined using a PCR that amplified part of the acuA gene (289 bp of 582 bp). Total DNA was extracted from bacteria grown overnight on Iso-Sensitest plates (Oxoid Ltd, Basingstoke, Hampshire, UK) at 30°C. Five bacterial colonies were suspended in 20 μl of lysis buffer (0.25% sodium dodecyl sulfate (SDS), 0.05 N NaOH). The suspension was heated for 15 min at 95°C. After addition of 180 μl of sterile H2O and centrifugation, supernatants were used for PCR experiments. Amplification reactions were carried out in a total volume of 25 μl containing 5 μl of total DNA, 25 pmol of each primer and 3 U of Taq DNA polymerase (Qiagen, Hilden, Germany). Primers for amplification of the acuA gene were acuA forward (5′-CAA CGC TAT GTG CTG CTG G-3′, located at position 32–50 of the acuA gene) and acuA reverse (5′-GGC CCA CCC AAA GTA ATC C-3′, located at position 320–302 of the acuA gene). Amplification conditions consisted of an initial cycle at 94°C for 1 min followed by 35 cycles of 30 s at 94°C, 40 s at 48°C, 1 min at 72°C, and a final cycle at 72°C for 6 min. PCR products were visualized by electrophoresis and staining with ethidium bromide on 1.5% agarose gels.

**Statistical analysis**
Results are means ± standard deviations unless indicated otherwise. Data were analyzed for statistical significance using the Wilcoxon rank sum test. p-Values of ≤0.05 were considered significant.

**Results**

**Adhesion to human bronchial epithelial cells and sheep erythrocytes**
To investigate whether CsuE-mediated pili are involved in the initial colonization process, adherence of *A. baumannii* 19606T and its isogenic CsuE-mutant #144 to human bronchial epithelial cells was compared in vitro. Light microscopy analysis of Giemsa-stained cells showed that the CsuE-mutant adhered to a significantly (p < 0.05) higher percentage of epithelial cells (29 ± 6.8%) than strain 19606T (16 ± 3.5%). The number of bacteria per
infected cell was not significantly higher for the mutant (1.9±0.2 bacteria/infected cell) than for the parental strain (1.4±0.1 bacteria/infected cell). Of note, the monolayers and morphology of the bronchial epithelial cells remained intact after 1 h incubation with either of the two *A. baumannii* strains. Differences in inoculum size between experiments did not influence the outcome of the adherence assays (data not shown).

SEM analysis revealed that strain 19606T and the CsuE-mutant were able to adhere to sheep erythrocytes (Fig. 1), showing that both strains can attach to at least two types of vertebrate cells.

![Fig. 1. Scanning electron micrographs of *A. baumannii* strain 19606T (A) and its CsuE-mutant (B) in the presence of sheep erythrocytes. Bars: 1 μm.](image)

**Cytokine induction in human bronchial epithelial cells**

To investigate whether CsuA/BABCDE-mediated pili contribute to the ability of *A. baumannii* to induce cytokine production by human cells, bronchial epithelial H292 cells were exposed to strain 19606T and its CsuE-mutant in vitro and the levels of IL-6 and IL-8 in the culture supernatant were determined after 24 h. The results revealed that bronchial epithelial cells co-incubated with strain 19606T or its CsuE-mutant produced significantly (p<0.05) higher levels of the cytokine IL-6 (875±398 pg/ml and 823±441 pg/ml, respectively) and the chemokine IL-8 (1751±608 pg/ml and 1930±705 pg/ml, respectively) than unstimulated cells (274±159 pg/ml IL-6 and 428±105 pg/ml IL-8). However, there was no significant difference between the ability of strain 19606T and the mutant to elicit IL-6 and IL-8 production by bronchial epithelial cells.

**Electron microscopy analysis of bacterial surface structures**

SEM analysis of bacteria cultured for 16 h at 30°C on blood agar plates revealed the existence of two types of cell appendages in strain 19606T: thin and short (median 29 nm; range 5–140 nm, n = 20 bacteria) pili-like structures and long cell extensions (Fig. 2A,B).
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The latter (median 260 nm, n = 20 bacteria) varied in length from 143 nm to 1008 nm and were irregularly distributed over the cell surface. The CsuE-mutant, which was grown under similar conditions, lacked the long cell extensions and had a more dense distribution of the short pili at its surface than strain 19606T (Fig. 2D). Of note, short pili could not be observed on all mutant cells. The CsuE-mutant adhered poorly to the poly-l-lysine-coated glass slides used for SEM and bacteria of this strain were mainly found adhering to debris on the glass slide. Interestingly, the short pili were seen mainly at the interface between the bacterial surface and the debris, suggestive of their involvement in adherence. Furthermore, the mutant strain showed, in contrast to the parental strain, only a few cell clusters with no more than two cells grouped together on the surface of the glass slides (Fig. 2C).

![Figure 2](image)

**Figure 2.** Scanning electron micrographs of *A. baumannii* 19606T (A and B) and CsuE-mutant (C and D) cells suspended from blood agar plates. Black arrows indicate long cell extensions; white arrows indicate thin and short pili. Bars: 1 μm (A and C) and 100 nm (B and D).

**Detection of CsuA/B protein**

Since secretion of CsuE, which is the tip adhesion on the pilus, is thought to precede the secretion of the CsuA/B subunit for pilus biogenesis [20], we reasoned that bacteria lacking this secretion event build up the CsuA/B protein in their cytoplasm, leading to
feedback inhibition or degradation of CsuA/B. We assessed the production of CsuA/B by *A. baumannii 19606<sup>T</sup>* and its CsuE-mutant by immunoblot analysis of whole lysates with anti-CsuA/B serum. The results demonstrated that the 18-kDa CsuA/B protein is expressed by the parental strain 19606<sup>T</sup>. In contrast, the isogenic CsuE-mutant did not express detectable levels of CsuA/B protein (Fig. 3), indicating that the pili produced by the CsuE-mutant are different from those assembled via the CsuA/BABCDE chaperone–usher system.

![Image](image.jpg)

**Figure 3.** Detection of the CsuA/B protein in *A. baumannii* 19606<sup>T</sup> (lane 1) and the CsuE-mutant (lane 2). M, molecular weight markers.

**Detection of acuA gene**

*A. baylyi* strain ADP1 expresses a chaperone usher system, named Acu, which is required for formation of thin pili in this environmental isolate [8]. To determine whether this system might be involved in formation of CsuA/BABCDE-independent short pili in *A. baumannii*, the presence of *acuA*, which encodes the structural subunit of thin pili in *A. baylyi* [8], was determined using PCR. Although not shown, this approach showed that a 289-bp *acuA* amplicon could be produced when total DNA from *A. baylyi* strain ADP1 was used as a template. In contrast, no amplicon was produced when total DNA from *A. baumannii* strain 19606<sup>T</sup> and the CsuE-mutant were used as a template. These observations suggest that the short pili produced by the parental strain 19606<sup>T</sup> and the CsuE-mutant are assembled by a system that seems different from CsuA/BABCDE and Acu.

**Discussion**

In this study, we demonstrated that production of the CsuA/BABCDE-mediated pilus, which is essential for biofilm formation on abiotic surfaces [22], is not required for the adherence of *A. baumannii ATCC19606<sup>T</sup>* to H<sub>292</sub> cells and induction of inflammatory cytokine production by these bronchial epithelial cells. Interestingly, in addition to the CsuA/BABCDE-mediated pilus, strain 19606<sup>T</sup> was found to produce CsuA/BABCDE-
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Our conclusion is based on the following findings. First, light and scanning electron microscopy showed that *A. baumannii* type strain 19606<sup>T</sup> and its isogenic CsuE-mutant adhered to human bronchial epithelial cells and sheep erythrocytes in vitro, indicating that CsxA/BABCDE-mediated pili are not essential for adherence to vertebrate cells. The finding that the CsuE-mutant strain adhered to a significantly larger number of epithelial cells than the parental strain might be attributed to increased expression of another type of pili on the surface of the latter strain, as has been described for *Escherichia coli* [15]. Secondly, we found that *A. baumannii* type strain 19606<sup>T</sup> and its CsuE-mutant induced similar levels of inflammatory cytokines IL-6 and IL-8 in bronchial epithelial cells, suggesting that CsxA/BABCDE-mediated pili are not involved in induction of inflammatory responses in human bronchial epithelial cells interacting with this bacterial pathogen.

The presence of pili on the surface of *Acinetobacter* strains was first described by Henrichsen and Blom in 1975 in *A. baylyi* BD4 (formerly *A. calcoaceticus* BD4) [9]. This strain was found to produce bundle-forming thin pili (2–3 nm in diameter) associated with adherence to abiotic and biotic surfaces [8] and individual thick pili (6 nm in diameter) involved in twitching motility [9], i.e., the ability to move on solid surfaces [14] enabling colonization of adjacent surface areas. The involvement of thin pili in adherence to abiotic surfaces was also demonstrated for *A. venetianus* strain RAG-1 [19]. Recently, Ishii et al. described two types of appendages in the highly adhesive *Acinetobacter* sp. strain Tol 5, an anchor-like appendage that tethers the bacterium to the substratum over distances of several hundreds of nanometers, and a peritrichate fibril-type appendage that attaches to the substratum at multiple places fixing the cell at shorter distances [10]. We have extended these findings by describing the existence of two types of cell appendages in the *A. baumannii* type strain 19606<sup>T</sup>: (i) irregular long cell extensions connecting bacteria that are assumed to represent the CsxA/BABCDE-mediated pili described by Tomaras et al. [22] and; (ii) thin and short pili-like structures that contact the surface areas adjacent to bacterial cells. The latter pili were not observed by Tomaras et al. [22], possibly because of the use of different experimental conditions and techniques, i.e., Luria–Bertani media and transmission electron microscopy versus blood agar and scanning electron microscopy, which were used to culture bacteria and visualize surface structures, respectively. Interestingly, the short pili were mainly seen at the site where the bacterium adhered to a surface area. Lee et al. [13] also described the presence of thin pili-like extensions on the surface of an *A. baumannii* strain that anchored the bacterium to the membrane surface of human bronchial epithelial cells. These findings raise the challenging question as to
whether the presence of a particular surface area triggers the production of short pili by *A. baumannii*, as has been described for *Acinetobacter* sp. strain Tol 5 [11].

It should be noted that although no PCR product could be amplified, *acuA*, which encodes pili in *A. baylyi* that are related to F17 pili of *Escherichia coli* [8], still could be present in *A. baumannii* AB307-0294. This is supported by the finding that a genome search of the *A. baumannii* AB307-0294 genome using *acuA* from *A. baylyi* leads to detection of genes encoding subunits of F17-related pili.

In summary, in addition to the already described CsuA/BABCDE-mediated pilus that plays a role in biofilm formation on abiotic surfaces, *A. baumannii* ATCC19606\(^{T}\) produces a CsuA/BABCDE-independent thin and short pilus, which may be involved in adherence of the bacterium to biotic surfaces such as those of human respiratory cells. Further genetic, structural and physiological studies are necessary to elucidate the role of this thin pilus in the pathobiology of *A. baumannii*.

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