Adherence and autoaggregation phenotypes of a
Burkholderia cenocepacia cable pilus mutant

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Abstract

Cable pili are unique peritrichous adherence organelles expressed by certain strains of the opportunistic human pathogen Burkholderia cenocepacia. Cable pili have been proposed to facilitate binding to human epithelial cells and mucin, and may play a role in the ability of B. cenocepacia to colonise the respiratory tract of compromised hosts. In this study, a genetic approach was undertaken to assess the role of cable pilin in mediating adherence as well as bacterial cell-cell interactions. The cblA gene, encoding the major pilin subunit, was insertionally inactivated, and the resulting mutant was shown to be blocked in CblA expression and in cable pilus morphogenesis. Although non-piliated, the cblA mutant was not defective in adherence to either porcine mucin or to cultured A549 human respiratory epithelial cells. Microscopic and flow cytometric analyses of B. cenocepacia cultures revealed that cable pilus expression facilitated the formation of diffuse cell networks, whereas disruption of cable pilus biogenesis enhanced autoaggregation and the formation of compact cell aggregates. Autoaggregation was observed both in culture and during B. cenocepacia infection of A549 epithelial cell monolayers. These findings indicate that cable pilus expression plays an important role in mediating B. cenocepacia cell-cell interactions, and that both cable pilus-dependent and cable pilus-independent mechanisms may contribute to B. cenocepacia adherence to cellular and acellular surfaces.

Keywords: Burkholderia cenocepacia; Cable pilus; Autoaggregation; Adherence; Flow cytometry

1. Introduction

Burkholderia cepacia is a complex of Gram-negative bacteria found ubiquitously in nature and is comprised of at least nine genomic species or genomovars [1–3]. Bacterial species belonging to the B. cepacia complex (Bcc) have received considerable attention, due to their ongoing development as agents for bioremediation and biological control [4] as well as their recent emergence as opportunistic human pathogens. Members of the Bcc colonise the lower respiratory tracts of immunocompromised individuals and particularly cystic fibrosis (CF) patients, generally causing chronic and sometimes fatal infections [5,6]. While isolates belonging to all nine genomovars of the Bcc have been cultured from CF patients, genomovar III strains, recently reclassified as Burkholderia cenocepacia, are the predominant Bcc respiratory pathogens in CF [3].

B. cenocepacia colonisation, as well as patient-to-patient transmission of the organism, has been associated with the expression of filamentous adherence organelles known as cable pili [7,8]. These structures are peritrichously expressed on the bacterial cell surface, and derive their name from their unique cable-like intertwined morphology [9]. Cable-piliated strains of B. cenocepacia have been shown to bind mucins [10], glycoprotein components of the airway surface fluid, which are particularly abundant in the CF lung due to poor mucociliary clearance [5]. Additionally, cable-piliated strains have been shown to bind to cytokeratin 13 (CK13), a predominantly cytoplasmic protein that may become exposed on the surfaces of epithelia during the course of chronic infection in CF [11]. Binding to both mucins and CK13 has been shown to be mediated by a 22-kDa pilus-associated adhesin [11,12]. It has recently been demonstrated that certain strains of B. cenocepacia that do not express cable pili can still adhere to CK13 [13]. Thus, cable pili do not appear to be
essential for binding to CK13, although their expression could facilitate exposure of the 22-kDa adhesin and its binding to CK13.

The *B. cenocepacia cbl* locus is comprised of at least five genes, designated *cblB*, *cblA*, *cblC*, *cblD* and *cblS*. The first four genes encode the structural and accessory components of the cable pilus biosynthesis pathway [13]. The *cblA* gene encodes the major structural subunit of cable pili [9], while *cblB*, *cblC* and *cblD* are predicted to encode the periplasmic chaperone, outer membrane usher, and minor structural subunit, respectively [13]. All four components of the cable pilus biosynthetic apparatus share high homology to components of the *Escherichia coli* CS1 family of pilus assembly pathways. The fifth gene in the *cbl* locus, designated *cblS*, is predicted to encode a new member of the sensor kinase family of bacterial two-component signal transduction systems.

A genetic analysis of cable pili has been hampered by the incredible antibiotic resistance of *B. cenocepacia* strains, which has limited the number of useful selectable markers for the generation of isogenic mutants blocked in cable pilus expression. Due to the lack of defined mutant strains, it has been difficult to assess the role of cable pili in *B. cenocepacia* adherence, and studies to date have relied on comparing non-isogenic strains of *B. cenocepacia* either expressing or not expressing cable pili. In this study, allelic exchange mutagenesis was used to insertionally inactivate the *B. cenocepacia cblA* gene, encoding the major structural subunit of cable pili. The *cblA* mutant was blocked in cable pilus biogenesis and was used to examine the role of cable pili in adherence to host cells and mucus. Further analysis of the *cblA* null strain revealed that cable pilus expression is an important determinant in mediating bacterial cell–cell interactions, a finding that may have implications towards an understanding of the mechanisms underlying *B. cenocepacia* adherence to both cellular and acellular surfaces.

### Materials and methods

#### 2.1. Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this work are listed in Table 1. *E. coli* strains were grown with aeration at 37°C in Luria–Bertani (LB) broth or on LB agar plates supplemented with ampicillin (100 µg ml⁻¹), tetracycline (12 µg ml⁻¹) or chloramphenicol (30 µg ml⁻¹) as necessary. *B. cenocepacia* strains were grown with aeration at 37°C in tryptic soy broth (TSB), or on LB or tryptic soy agar (TSA) plates supplemented with chloramphenicol (300 µg ml⁻¹) as necessary.

#### 2.2. DNA manipulations

DNA-modifying enzymes, including restriction endonucleases, T4 DNA ligase, and T4 polymerase, were obtained from Roche, New England Biolabs and Invitrogen. Plasmid DNA was isolated by the boiling lysis method [14] or using the QIAprep Spin Miniprep kit (Qiagen Inc.). Recombinant plasmids were introduced into *E. coli* by electroporation using a Gene Pulser II (Bio-Rad). Genomic DNA from *B. cenocepacia* was extracted using the PurePrep S. Typhimurium miniprep kit (Qiagen Inc.). Southern blot hybridisations were generally performed as described by Sambrook et al. [14] using Hybond N nitrocellulose membranes and probes labelled with [α-³²P]dCTP (Amersham Pharmacia Biotech) by the random primer method.

#### 2.3. Construction of an isogenic *B. cenocepacia cblA* mutant

The *B. cenocepacia cblA* gene was amplified by polymerase chain reaction (PCR) from *B. cenocepacia* using Taq polymerase (Promega) and oligonucleotide primers *cbl1* and *cbl2*, based on the published *cblA* sequence [9].

### Table 1: Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td>E. coli strains</td>
<td></td>
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</tr>
<tr>
<td>DH5α</td>
<td><em>supE44 lacU169 (q80 lacZAM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</em></td>
<td>Bethesda Research Laboratories [37]</td>
</tr>
<tr>
<td>S17-1</td>
<td>Integrated RP4-2, Tc::Mu, Km::Tn7</td>
<td></td>
</tr>
<tr>
<td>B. cenocepacia strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BC7</td>
<td>CF clinical isolate, genomovar III, cable-piliated, formerly designated PC7</td>
<td>[9]</td>
</tr>
<tr>
<td>CM256</td>
<td><em>cblA::cat</em> derivative of BC7</td>
<td>This study</td>
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<tr>
<td>Plasmids</td>
<td></td>
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<tr>
<td>pBluescript SK⁺</td>
<td>Cloning and single-stranded phagemid; Ap⁺</td>
<td>Stratagene</td>
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<tr>
<td>pGEM-T Easy</td>
<td>TA cloning vector; Ap⁺</td>
<td>Promega</td>
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<tr>
<td>pCAT1</td>
<td>Source of <em>cat</em> cassette; Cm⁺</td>
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<tr>
<td>pNPT138</td>
<td>Derivative of pLITMUS18 cloning vector with nptI, RK2 oriT, and <em>Bacillus subtilis</em> sacB; Km⁺</td>
<td>M.R.K. Alley</td>
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<tr>
<td>pCMT2</td>
<td>Tetracycline-resistant derivative of pNPT138; Tc⁺</td>
<td>This study</td>
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<tr>
<td>pMT4</td>
<td>779-bp <em>cblA</em> PCR product cloned in pGEM-T Easy; Ap⁺</td>
<td>This study</td>
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<tr>
<td>pMT5</td>
<td>1.5-kb <em>cblA::cat</em> construct derived from pMT4; Ap⁺, Cm⁺</td>
<td>This study</td>
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<tr>
<td>pMT6</td>
<td>1.5-kb <em>cblA::cat</em> EcoRI fragment subcloned into pCMT2; Cm⁺</td>
<td>This study</td>
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sequence of primer cbl1 is 5’-AATGGGCAGATGTGCAGCAG-3’, and the sequence of primer cbl2 is 5’-CGCGATGTTCCATCACATAC-3’. The PCR reactions were carried out for 35 cycles consisting of 94°C (1 min), 55°C (1 min) and 72°C (1 min), concluding with an additional 5 min extension time at 72°C at the end of the 35 cycles. The 779-bp PCR product encoding the entire cblA gene was cloned into the pGEM T-Easy TA cloning vector (Promega) to generate pMT4. The cblA gene in pMT4 was insertional inactivated by introducing the chloramphenicol acetyltransferase gene (cat), cloned as a HindII fragment into the Smal site in cblA, generating plasmid pMT5. The 1.5-kb cblA::cat construct was cloned into the suicide vector pCMT2 as an EcoRI fragment (pMT6) and delivered to B. cenocepacia by conjugation, using E. coli strain S17-1 as the donor. B. cenocepacia recombinants were selected on LB agar plates containing chloramphenicol and kanamycin to select against wild-type B. cenocepacia and the E. coli donor strain, respectively. Double crossover mutants were confirmed by PCR and Southern analysis using both cblA and cat as probes. The cblA null mutant was designated CM256.

2.4. Electron microscopy

B. cenocepacia strains grown on LB agar plates were used to inoculate TSB and grown with shaking for 17 h. The cultures were removed and allowed to settle for 2 h at room temperature without shaking. The unsettled population of the wild-type strain BC7 culture was found to be enriched for cable-piliated cells. Aliquots were taken from the unsettled cultures and applied onto formvar-coated grids. Excess liquid was removed by blotting. The bacterial cells were stained with 0.5% uranyl acetate and analysed in grids. Excess liquid was removed by blotting. The bacterial cells were harvested by centrifugation, briefly washed with 70% ethanol, resuspended in 6 ml of phosphate-buffered saline (PBS) and cells were vortexed vigorously to shear the cable pili. The bacterial cells were harvested by centrifugation, briefly washed with 70% ethanol, resuspended in Laemmli buffer and analysed by immunoblotting as previously described [16]. Equivalent amounts of protein were loaded on 12.5% SDS–PAGE. The blots were probed with the primary CblA antiserum at a dilution of 1:10,000, and the secondary antibody (goat anti-rabbit IgG, conjugated to horseradish peroxidase) at the same dilution. Western blots were developed with the Renaissance chemiluminescence kit (DuPont NEN), and exposed to X-ray film (Kodak).

2.8. Porcine mucin adherence assays

Porcine gastric mucin used in adherence studies was obtained from Sigma-Aldrich. Mucin was dissolved at a concentration of 50 µg ml⁻¹ in water, filter-sterilised, and 50-µl aliquots were added to wells of a 96-well microtitre plate. To coat the wells, plates were incubated at 37°C for 18 h. Stationary-phase cultures of B. cenocepacia strains grown in TSB were resuspended in PBS to an OD₆₀₀ of 1.0 (approximately 8 × 10⁸ CFU ml⁻¹). For the adherence assay, 50-µl aliquots of bacterial suspensions were added to mucin-coated wells. After 30 min of incubation, wells were washed 15 times with 200 µl PBS to remove unbound bacteria, and the bound bacteria released by the addition of 200 µl of 0.25% Triton X-100. The bound bacteria were enumerated by plating serial dilutions on LB agar. Mucin adherence assays were performed in triplicate, with two independent enumerations for each well.

2.9. A549 adherence assays

Assays to examine binding of B. cenocepacia strains to cultured A549 human respiratory epithelial cells were performed essentially as previously described [17]. Briefly, B. cenocepacia strains were grown in TSB to an OD₆₆₀
of ~0.6, harvested by centrifugation, resuspended in RPMI 1640 media lacking foetal bovine serum (FBS). Bacteria were added to A549 monolayers, which were seeded at 5 × 10² per well into a 24-well tissue culture plate 24 h prior to the adherence assay, at a multiplicity of infection (MOI) of approximately 10:1 or 100:1. The tissue culture plate was centrifuged to bring bacterial cells in contact with the A549 monolayers, followed by 1 h of incubation at 37°C in the presence of 5% CO₂. The non-adherent bacteria were removed from the wells by washing five times with PBS, and the adherent bacteria were released by lysing the A549 monolayers with 0.25% Triton X-100. Adherent bacteria were enumerated by plating serial dilutions of lysates on LB agar and viable bacterial cell counts. Adherence assays were performed in triplicate, with two independent enumerations for each well.

To visualise the adherence patterns of the wild-type *B. cenocepacia* strain BC7 and the cblA null strain, bacteria were labelled with fluorescein isothiocyanate (FITC) as described by Weingart et al. [18]. Briefly, mid-exponential phase cultures (OD₆₀₀ ~0.6) were harvested, resuspended in a solution of FITC (Sigma Aldrich; 0.5 mg ml⁻¹) in 50 mM sodium carbonate–100 mM sodium chloride, pH 8.0, and incubated 20 min. Unbound FITC was removed by washing the bacteria three times with PBS, and the bacteria were resuspended in RPMI 1640 media lacking FBS. The FITC-labelled bacteria were added to monolayers of A549 human respiratory epithelial cells, seeded 24 h prior to the experiment at 5 × 10⁵ cells per well in a 24-well plate, at a MOI of 100:1. After centrifugation, bacteria were allowed to adhere for 1 h at 37°C, as for the quantitative adherence assay, after which non-adherent bacteria were removed by five consecutive washes with PBS, followed by the addition of 500 μl of RPMI 1640 with 10% FBS to each well. Light and fluorescence microscopy were performed using a Nikon Eclipse TE200 microscope and a 40× lens. Bacterial cells were visualised using fluorescence microscopy, and the A549 human respiratory cells were visualised using standard phase-contrast microscopy. The bacterial cells were coloured green for ease of visualisation, and the corresponding phase-contrast and fluorescence images were overlayed for each field of view examined.

2.10. Quantitative aggregation assay

*B. cenocepacia* strains BC7 and CM256 (cblA::cat) were grown in TSB for 17 h at 37°C with aeration. These cultures were used to inoculate fresh TSB at a dilution of 1:100 and grown for an additional 17 h. The optical densities of the cultures were determined and equilibrated by the addition of fresh TSB as necessary. The absorbances of each culture were continually measured at 600 nm in 4-ml acrylic cuvettes (Sarstedt Inc.) using an Ultrospec 2000 spectrophotometer (Pharmacia Biotech). The assay was performed in triplicate over a time course of 8 h.

2.11. Light microscopy

For light microscopy, stationary-phase cultures were used to inoculate 3-ml aliquots of fresh TSB and incubated for 20 h at 37°C with aeration. The cultures of the wild-type and cblA mutant strains were gently diluted 1:10 in PBS prior to analysis. To examine the unsettled population of the wild-type strain BC7, a 20-h culture was incubated without agitation for an additional 6 h at room temperature, upon which the supernatant was analysed by microscopy. All samples were examined by an Olympus BX60 microscope at a magnification of 600 ×.

2.12. Flow cytometry

Stationary-phase *B. cenocepacia* cultures grown in TSB were used to inoculate 3-ml aliquots of fresh TSB media. The strains were grown for 20 h at 37°C with aeration, diluted 1:10 in PBS with gentle mixing, and analysed on a Becton Dickinson FACSscan. The forward scatter profiles of 10,000 particles were analysed for each sample. As a baseline for the single-cell population, a sample of *B. cenocepacia* strain BC7 was vortexed vigorously prior to flow cytometric analysis. To examine aggregation in the unsettled subpopulation of wild-type *B. cenocepacia* cultures, stationary-phase BC7 cultures were incubated for 6 h without shaking, and their supernatants analysed by flow cytometry as described above. Data analysis was performed using CellQuest version 3.3 software (Becton Dickinson).

3. Results

3.1. Generation of a cblA null mutant

We have previously demonstrated the utility of the chloramphenicol acetyltransferase (*cat*) gene of *Campylobacter coli* for the generation of targeted gene disruptions in *B. cenocepacia* [17]. In order to further define the role of the *cbl* locus in cable pilus morphogenesis, we utilised the *cat* gene to generate a *cblA* null mutant in strain BC7, a cable-piliated CF clinical isolate of *B. cenocepacia* [9]. The *cblA* gene was cloned and insertionally inactivated by the introduction of the chloramphenicol resistance cassette (*cat*) into the *cblA* coding region. The insertionally inactivated copy of the *cblA* gene was delivered to *B. cenocepacia* strain BC7 on a suicide plasmid via conjugation, and used to replace the wild-type chromosomal copy by allelic exchange. Insertional inactivation of the chromosomal *cblA* gene by a double crossover event was confirmed by PCR and Southern analysis (data not shown). The *cblA* null mutant was designated CM256.
3.2. Characterisation of the cblA mutant

Examination of the wild-type B. cenocepacia strain BC7 by transmission electron microscopy (TEM) revealed numerous peritrichously expressed cable pili on the bacterial cell surface (Fig. 1A–D), consistent with previously published reports [9]. Often, the outwardly projecting pili appeared to tether adjacent cells together and mediate diffuse cell–cell interactions (Fig. 1C,D). While some cells in the wild-type B. cenocepacia strain BC7 culture were heavily piliated, the level of piliation was highly variable, with the majority of cells exhibiting only a few or no detectable pili. In contrast to the wild-type strain, cable pili were never observed on cells of the cblA mutant (Fig. 1E,F), confirming that insertional inactivation of the cblA gene leads to a block in cable pilus biogenesis.

To further characterise the non-piliated phenotype of the cblA mutant, semi-pure cable pilin fractions were prepared from wild-type B. cenocepacia strain BC7 and the isogenic cblA null strain (CM256), and analysed by SDS-PAGE. An abundant protein of approximately 15 kDa, corresponding in size to mature CblA, was detected in the semi-pure pilin fraction obtained from wild-type B. cenocepacia strain BC7 (Fig. 2A, lane 1). To confirm its identity as CblA, the 15-kDa protein was purified, and

Fig. 1. Transmission electron micrographs of the wild-type B. cenocepacia strain BC7 (A–D) and the cblA null strain CM256 (E,F). Scale bars indicate a length of 0.5 μm.

Fig. 2. Biochemical analysis of CblA expression by B. cenocepacia strain BC7 and the isogenic cblA mutant. A: Coomassie-stained SDS-PAGE gel of semi-pure cable pilin preparations from B. cenocepacia wild-type strain BC7, and strain CM256. Equal amounts of protein were loaded. Lanes: M, low-range molecular mass marker; 1, wild-type strain BC7; 2, CM256 (cblA::cat). The arrow denotes the position of the 15-kDa B. cenocepacia CblA major pilin. B: Immunoblot of whole-cell preparations of B. cenocepacia wild-type strain BC7 (lane 1), and CM256 (cblA::cat, lane 2), probed with the CblA-specific antiserum. Equivalent amounts of protein were loaded in each lane. The arrow denotes the position of the 15-kDa B. cenocepacia CblA major pilin.
its N-terminal amino acid sequence determined. The first 10 amino acids of the 15-kDa protein were identical to the previously published sequence of the mature CblA protein (VQKDITVTAN) [9]. In contrast to the wild-type B. cenocepacia protein sample, the 15-kDa CblA protein was not detected in semi-pure pilin fractions generated from strain CM256 (Fig. 2A, lane 2), consistent with the defect in pilus expression observed by TEM.

To confirm that the cblA null strain was blocked not only in export of CblA, but also in its expression, whole-cell extracts of the wild-type B. cenocepacia strain BC7 and strain CM256 were probed with CblA-specific antiserum. A 15-kDa band, corresponding in size to the CblA protein, cross-reacted with the CblA-specific antiserum in the wild-type strain BC7 whole-cell preparation, but not in the strain CM256 sample (Fig. 2B), confirming that the cblA mutant is blocked in CblA expression. Together, these results confirm that disruption of cblA in B. cenocepacia strain BC7 results in a block in cable pilus biogenesis.

3.3. Adherence of the cblA mutant to porcine mucin and cultured A549 human respiratory epithelial cells

Previous studies have shown that cable-piliated strains of B. cenocepacia can bind to mucin of both human and porcine origin [10,19]. However, these studies were limited to using non-isogenic strains of B. cenocepacia as non-piliated controls. To examine the role of cable pili in binding to mucin, the abilities of the wild-type B. cenocepacia strain BC7 and the isogenic cblA mutant (CM256) to adhere to porcine mucin-coated microtitre plates were compared. Insertional inactivation of the cblA gene did not inhibit binding to porcine mucin, as there was no statistically significant difference in binding of the two strains (Fig. 3A, \( P = 0.39 \)).

We and others have previously shown that B. cenocepacia can adhere to, and invade, cultured A549 human respiratory epithelial cells [17,20–23]. To determine whether adherence to A549 cells is dependent on cable pili expression, binding of the wild-type B. cenocepacia strain BC7 and the cblA null strain to cultured A549 cells was compared. Bacteria were added to confluent A549 monolayers. For binding to remain in suspension, we hypothesised that the unsettled population of a wild-type BC7 culture would be more piliated than the settled cell population, and thus should have a greater amount of CblA. To examine this, a stationary-phase culture of B. cenocepacia strain BC7 was allowed to settle for 6 h, after which the unsettled and settled cells were harvested and analysed by immunoblotting. An abundant band corresponding to the CblA protein was observed in the unsettled population sample (Fig. 4B, lane U), while in the settled population, the CblA protein was detectable only upon overexposure (Fig. 4B, lane S, data not shown). These results indicate that unsettled cells are more cable-piliated than settled cells, and are consistent with the variable piliation of the wild-type strain BC7 cells observed by TEM.

Flow cytometry was also used to further analyse and quantify the observed differences in aggregation of B. cenocepacia strains. Aggregates of cells, due to their larger size, yield higher forward light scattering values in the flow cytometer than single cells. In order to establish the
baseline profile of a single-cell population, a sample of a BC7 culture was vortexed vigorously to disrupt the cell aggregates. The vortexed sample yielded a single peak when analysed by flow cytometry, with a mean forward scatter value (FSC-H) of approximately 10 units (Fig. 4C).

This peak was designated the single-cell population. When the unsettled population of strain BC7 was analysed by flow cytometry, it yielded a forward scatter profile similar to that of the vortexed culture, exhibiting a single peak corresponding to the single-cell population (Fig. 4C).

When a culture of the wild-type strain BC7 was analysed without vortexing, the majority of the counts were found in the single-cell population. However, some aggregation of the wild-type BC7 cells was observed, as demonstrated by a second peak with a higher forward scatter value of approximately 200 units (Fig. 4C). This peak was designated the aggregated cell population. When examined under the same conditions, cultures of the cblA mutant (CM256) yielded forward scatter profiles distinct from that of the wild-type strain, exhibiting a decrease in the number of counts in the single-cell population, accompanied by a pronounced increase in the aggregated cell population (Fig. 4C). Furthermore, the peaks corresponding to the aggregated cell population in the profile of strain CM256 were detected at higher forward scatter values than in the wild-type strain BC7, suggesting an increase in the size of aggregates in the cblA mutant culture (Fig. 4C).

To quantify the differences in aggregation between the wild-type strain BC7 and strain CM256, the mean percent of counts and mean forward scatter values of both the single and aggregated cell populations were determined from a total of four independent experiments. For the wild-type strain BC7 the percent of counts in the single-cell population was greater than the percent of counts in the aggregated cell population, while for the cblA mutant strain, the percent of counts was greater in the aggregated cell population (data not shown). When the mean forward scatter values in the single-cell population were compared, they were similar for wild-type BC7 and CM256, confirming that the cells of the cblA mutant are not abnormal in size. Moreover, the mean forward scatter values for the aggregated cell population of strain CM256 were approximately two-fold higher than that of the wild-type strain.

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**Fig. 4.** Differential aggregation of the wild-type *B. cenocepacia* strain BC7 and the isogenic cblA mutant. A: Quantitative aggregation assay. The changes in the absorbances (A<sub>600</sub>) of wild-type BC7 (black) and CM256 (cblA::cat; white) cultures were monitored over a period of 8 h, as described in Section 2. The bars represent standard errors of the mean. Inset: cultures of strain BC7 and CM256 after 8 h of incubation without shaking. U, unsettled population; S, settled population. B: Immunoblot of whole-cell preparations from unsettled (U) and settled (S) populations of a wild-type strain BC7 culture after 6 h of incubation without shaking, probed with the CblA-specific antiserum. Equal amounts of protein were loaded in each lane. The arrow indicates the CblA protein. C: Representative flow cytometric analysis of cultures of *B. cenocepacia* wild-type (wt) strain BC7, and the cblA mutant (CM256). The forward scatter readings (FSC-H) are indicated on the x-axis, and the number of counts recorded, on the y-axis. The single-cell population, determined from the analysis of the vortexed BC7 culture, and the aggregated cell population are denoted.
BC7 (data not shown), demonstrating that the cblA mutant formed aggregates on average twice the size of those formed by the wild-type strain BC7.

Microscopic examination of cultures of the cblA mutant strain (CM256) revealed the presence of aggregates of B. cenocepacia cells (Fig. 5C,D). Aggregates were also observed in the cultures of the wild-type strain BC7 (Fig. 5A,B), but they appeared to be less numerous and smaller in size than those observed in strain CM256 cultures. Furthermore, the unsettled population of the wild-type BC7 culture, enriched for cable-piliated cells, generally did not form compact aggregates (Fig. 5E,F). Instead, the piliated cells were dispersed into patterns resembling those observed by TEM, whereby cable pili were found to mediate diffuse cell–cell interactions (Fig. 1C,D). Together, the microscopy and flow cytometry data indicate that the enhanced ability of cable-piliated cells to remain in suspension is due to the more diffuse nature of cell–cell interactions mediated by cable pili as well as an inhibition in the ability of B. cenocepacia cells to autoaggregate.

3.5. B. cenocepacia autoaggregation during infection of A549 human respiratory epithelial cells

To determine whether the autoaggregation phenotype of the cblA mutant observed in culture could also be observed during B. cenocepacia infection of epithelial cells, A549 cell monolayers were infected with FITC-labelled strain BC7 or the cblA null strain (CM256). Following 1 h of incubation and repeated washing of the infected A549 cell monolayers, the bound bacteria were examined by fluorescence microscopy. Aggregates of B. cenocepacia cells were observed on the epithelial cell monolayers infected with either the wild-type strain BC7 or strain CM256 (Fig. 6A,B). Few aggregates were observed when the epithelial cell monolayers were infected at an MOI of 10:1 as compared to an MOI of 100:1, indicating that the aggregates observed were formed after adherence to the epithelial cell monolayers and not during culture of the bacteria (Fig. 6; data not shown). These findings demonstrate that the autoaggregation phenotype is not strictly associated with B. cenocepacia growth in broth, and that aggregation can also occur on a biological surface. Furthermore, these observations are also consistent with the microscopic and flow cytometric analyses, which together demonstrated that both the wild-type and cblA mutant cells can aggregate, and that autoaggregation preferentially occurs in the absence of cable pili.

**Fig. 5.** Micrographs of cultures of the wild-type strain BC7 (A,B), the cblA mutant strain CM256 (C,D) and the unsettled population of strain BC7 (E,F). The arrows indicate B. cenocepacia cell aggregates. Scale bars indicate a length of 5 μm.

**Fig. 6.** Adherence patterns of the wild-type B. cenocepacia strain BC7 (A) and the cblA null strain CM256 (B) during infection of A549 cell monolayers. Infected A549 cells and FITC-labelled bacteria were visualised at 1 h post-infection by phase-contrast and fluorescence microscopy, as described in Section 2. Scale bars indicate a length of 10 μm.
4. Discussion

A number of studies have demonstrated that cable-piliated strains of B. cenocepacia can adhere to mucin as well as human buccal, bronchial, and respiratory epithelial cells [9–11,19,23,24]. In addition to a role in adherence, there is also evidence that cable pilus expression can influence B. cenocepacia multicellular behaviour [9,23]. In order to further define the role of cable pili in adherence as well as mediating bacterial cell–cell interactions, we generated an isogenic cblA mutant of the cable-piliated B. cenocepacia strain BC7. The cblA mutant was shown to be blocked in cable pilus morphogenesis and in expression and export of CblA, the major structural subunit of cable pili.

Adherence assays using porcine mucin and A549 human respiratory epithelial cells did not detect a significant difference in binding between the wild-type B. cenocepacia strain BC7 and the isogenic cblA mutant. These findings suggest that B. cenocepacia expresses other factor(s) that can participate in binding to both mucin and A549 cells. Strain BC7, as well as other cable-piliated strains of B. cenocepacia, can express a 22-kDa adhesin protein that has been shown to mediate binding to bronchial epithelial cells and CK13 [11]. The indistinguishable binding profiles of the wild-type and cblA null strains may be due, in part, to the expression of the 22-kDa adhesive in the cblA mutant strain, which may mediate binding in the absence of cable pili. Additionally, B. cenocepacia may express other adhesins that can participate in the binding to both mucin and A549 cells, which may mask any direct role of cable pili in adherence. We also cannot rule out the possibility that a significant difference in binding between the wild-type and cblA null strains was not measured due to limitations in the use of porcine mucin and cultured A549 cells to examine B. cenocepacia adherence. Adherence assays using human mucin, which cable-piliated strains have been shown to bind with high affinity, or the use of human cells highly expressing CK13, may reveal a measurable difference in binding between the wild-type strain BC7 and the cblA mutant. The establishment of such assays in our laboratory is currently underway.

It has previously been reported that distinct cable-piliated isolates of B. cenocepacia exhibit a wide range of piliation levels [12]. However, the degree of heterogeneity in cable pilus expression observed in this study within cultures of a single strain was surprisingly high. Although some of the cells of the B. cenocepacia wild-type strain BC7 were heavily piliated, the majority expressed few or no pili on their surface. Future studies will determine whether variable cable pilus expression in B. cenocepacia is due to a phase variation mechanism, as has been characterised for type I and P pili of E. coli [25,26], or a different process.

When incubated in the absence of shaking, we observed a difference between cultures of the wild-type B. cenocepacia strain BC7 and the isogenic cblA null strain. This difference was manifested by an increase in the rate of settling of the cblA mutant culture. In contrast, expression of cable pili on the surfaces of the wild-type B. cenocepacia cells appeared to prevent settling of cultures. Microscopy and flow cytometric analyses revealed the presence of aggregates of cells in the cultures of the cblA mutant strain. These aggregates were on average twice the size of those formed in cultures of the wild-type strain BC7. The clusters of cells formed through cable pilus interactions in unsettled cultures of the wild-type strain appeared more diffuse, with B. cenocepacia cells tethered at a distance from one another. Together, our results indicate that the enhanced ability of cable-piliated cells to remain in suspension is due to an inhibition in the ability of B. cenocepacia to autoaggregate as well as the more diffuse nature of cell–cell interactions mediated by cable pili. Although we have not completely ruled out possible polar effects of the cblA mutation, the aggregation phenotype of the cblA mutant is likely due to the block in pilus biogenesis and not a secondary effect of the mutation, as we have demonstrated that even subpopulations of the wild-type strain which are reduced in levels of CblA protein and piliation are also prone to forming aggregates (Figs. 4 and 5).

The autoaggregation phenotype observed in culture was also observed during B. cenocepacia infection of A549 cell monolayers. These aggregates were still present even after repeated washing of the infected cell monolayers, suggesting a potential role for autoaggregation in cooperative binding to host cells. Chiu et al. [23] recently reported that a cable-piliated B. cenocepacia strain (C5424) exhibited clump formation when bound to A549 cells, while a non-piliated strain of B. cenocepacia predominantly showed single-cell binding, together suggesting that cable pili promote autoaggregation. Since a non-isogenic strain of B. cenocepacia was used as a non-piliated control in these studies, the role of cable pili in mediating the clumping behaviour of strain C5424 remains to be defined. Our studies, using an isogenic cblA mutant, argue against a role for cable pilus-mediated formation of compact cell aggregates, and provide evidence that autoaggregation is actually enhanced when cable pilus expression is blocked.

Pili expressed by other bacterial species, such as type IV pili of Neisseria gonorrhoeae [27], Neisseria meningitidis [28] and Vibrio cholerae [29], and the bundle-forming pili of enteropathogenic E. coli [30], have been implicated in facilitating the formation of compact aggregates of cells. Interestingly, we have observed the opposite effect, whereby autoaggregation of B. cenocepacia cells preferentially occurs in the absence of cable pili. Similar findings have been reported in E. coli, where autoaggregation mediated by the antigen 43 protein (Ag43) is abrogated by expression of type I fimbriae [31]. Furthermore, expression of E. coli type I and P pili has been shown to block the expression of Ag43 [32]. It is possible that the physical presence of cable pili on the B. cenocepacia cell surface, which has been shown to mediate diffuse cell–cell interac-
tions, prevents the contact required for autoaggregation to occur. In addition, cable pilus expression may inhibit expression of the factor(s) mediating autoaggregation.

Our characterisation of both cable pilus-dependent and cable pilus-independent cell–cell interactions may have implications towards an understanding of the pathogenesis of *B. cenocepacia* in CF. The diffuse nature of cell clusters formed by cable pili may allow greater exposure of the cable pilus adhesin(s) to the environment, increasing accessibility of the 22-kDa pilus-associated adhesin to CF lung mucins and CK13 on epithelial cells, and thus facilitating colonisation. It is conceivable that autoaggregation, independent of cable pilum, may also contribute to *B. cenocepacia* pathogenesis by mediating bacterial cell–cell interactions and cooperative adherence to host receptors.

Proteins mediating autoaggregation in other bacterial species have been well characterised, including the filamentous haemagglutinin of *Bordetella* spp. [33], Ag43 of *E. coli* [34], M proteins of group A streptococci [35], and YadA of *Yersinia enterocolitica* [36]. It is possible that the 22-kDa adhesin, which appears to be cable pilus-associated in those strains that express cable pilum, may mediate autoaggregative behaviour in their absence. Alternatively, other protein(s) or non-proteinaceous molecule(s), which remain to be identified, are responsible for compact cell–cell interactions in *B. cenocepacia*. Ongoing studies in our laboratory will continue to define the elements mediating such interactions.

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