Lack of cable pili expression by *cbIA*-containing *Burkholderia cepacia* complex

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The *Burkholderia cepacia* complex consists of several closely related bacterial species (or genomovars) which although generally not pathogenic for healthy individuals, contribute significantly to morbidity and mortality among persons with cystic fibrosis (CF). Certain *B. cepacia* complex strains are more frequently recovered from CF sputum cultures than are others, and these typically reside in genomovar III. The ET12 clone is a genomovar III strain that predominates among CF patients in Canada and the United Kingdom and is characterized by distinctive *cbIA*-encoded pili that have a cable-like morphology. In a previous survey of *B. cepacia* complex isolates recovered from 606 CF patients in the US, a single genomovar III ET12 isolate (isolate AU0007) was identified; several *cbIA*-containing genomovar I isolates, however, were also detected. In the study reported here, analysis by PFGE revealed several distinct strain types among these genomovar I isolates, and sequence analysis of their *cbIA* genes demonstrated 87.8–88.4% identity to the ET12 *cbIA* sequence. Southern analysis indicated that the *cbIA* variant from each genomovar I isolate resides on a 4 kbp *EcoRI* fragment, in contrast to ET12 isolates, in which *cbIA* localizes to a 5 kbp *EcoRI* fragment. Western blot assay indicated expression of the 16 kDa major pilin subunit by ET12 isolates, including AU0007, but neither whole-cell nor surface-protein extracts of the genomovar I reacted. Electron microscopy revealed the complete absence of pili expression by the genomovar I isolates. In contrast to typical ET12 isolates, AU0007 appeared to be hyperpiliated with rigid pili that lacked the cable morphology and did not bind cytokeratin 13, which has been previously identified as the epithelial cell receptor for the ET12 cable-pili-associated adhesin.

Keywords: *cbIA* gene, bacterial adherence, cystic fibrosis

INTRODUCTION

The *Burkholderia cepacia* complex consists of several phylogenetically closely related yet distinct species of soil commensal and phytopathogenic bacteria (Coenye *et al.*, 2001). New binomial designations have been assigned to some species within this group (i.e. *B. multivorans*, *B. stabilis*, *B. vietnamiensis*, *B. ambifaria*, *B. anthina* and *B. pyrrocinia*), while the remaining species continue to be referred to as genomovars (i.e. *B. cepacia* genomovars I, III and VI) pending the identification of species-specific phenotypic characteristics.

Abbreviations: CF, cystic fibrosis; CK13, cytokeratin 13.

The GenBank accession numbers for the complete *cbIA* nucleotide sequences for the isolates listed in Table 1 are AF455151–AF455162.

Ultimately, the name *B. cepacia* will be restricted to genomovar I, the species that contains the original species type strain.

Certain *B. cepacia* complex strains have attracted considerable attention because of their potential commercial use as biopesticidal and bioremedial agents (LiPuma & Mahenthiralingam, 1999; Parke & Gurian-Sherman, 2001). At the same time, there has been a growing appreciation of the role *B. cepacia* complex species play as opportunistic human pathogens. Although generally not pathogenic for healthy humans, these species are capable of causing severe infection in certain vulnerable populations, particularly persons with cystic fibrosis (CF), the most common lethal genetic disease among whites (LiPuma, 1998). In CF, respiratory tract infection with *B. cepacia* complex is associated

with increased rates of morbidity and mortality. In fact, following infection, a significant proportion of CF patients will die, with rapidly progressive necrotizing pneumonia and sepsis.

The bacterial virulence factors and pathogenic mechanisms involved in human infection due to *B. cepacia* complex remain to be elucidated. Nevertheless, it is clear that some specific strains are more frequently recovered from CF patients than are others. One such 'epidemic' strain, the ET12 lineage, is a genomovar III strain that predominates among patients in Ontario, Canada, and is found in about one-third of *B. cepacia*-infected patients in the United Kingdom (Pitt *et al.*, 1996; Sun *et al.*, 1995). This strain expresses a distinctive pilus (the *cblA*-encoded cable pilus) and an associated adhesin that mediate bacterial binding to CF respiratory epithelium (Sajjan *et al.*, 1995, 2000a, b). The cognate receptor for cable-pili expressing *B. cepacia*, cytokeratin 13 (CK13), is a 55 kDa protein that is enriched in CF epithelia (Sajjan *et al.*, 2000a). Because the expression of cable pili may provide an important pathogenic mechanism contributing to inter-patient spread of *B. cepacia* complex, differential infection control measures based on the presence (or absence) of *cblA* have been proposed (Clode *et al.*, 2000).

In a recent study only one of 606 *B. cepacia* complex-infected US CF patients harboured a *cblA*-containing ET12 strain (LiPuma *et al.*, 2001). Analyses employing *cblA*-specific dot-blot and PCR assays, and genotyping by PFGE indicated, however, that nine additional patients were infected with non-ET12 strains also positive for *cblA* sequences. An additional *cblA*-positive isolate was identified from stream sediment. Interestingly, all 10 of these *cblA*-positive, non-ET12 isolates were *B. cepacia* genomovar I, a species not frequently found in CF sputum culture (LiPuma *et al.*, 2001). In this study we investigated the expression of cable pili by these strains and characterized the *cblA* gene variants found therein.

METHODS

Bacterial strains with *cblA* sequences. Bacterial strains were obtained during a study of *B. cepacia* complex recovered from persons with CF as previously reported (LiPuma *et al.*, 2001). In that study, 10 of 606 isolates (from 606 CF patients receiving care in 132 treatment centres in 105 US cities) were identified by dot-blot hybridization as containing genomic DNA sequences homologous to *cblA*. By using 16S rDNA- and *recA*-based species-specific PCR assays (LiPuma *et al.*, 1999; Mahenthiralingam *et al.*, 2000a) one of these isolates (AU0007) was found to be genomovar III whereas the remaining nine were identified as genomovar I. For the present study, an additional isolate with *cblA* sequence (BC20) was identified from among *B. cepacia* complex isolates recovered from stream sediment (Wise *et al.*, 1995); this too was identified as genomovar I. BC7 (Sajjan *et al.*, 1995; Mahenthiralingam *et al.*, 2000b) and J2315 (Govan *et al.*, 1993; Mahenthiralingam *et al.*, 2000b) are representatives of the genomovar III ET12 lineage. Although both isolates have been shown in previous work to contain *cblA* genes, J2315 lacks the 22 kDa adhesin and does not bind to CK13 (Sajjan *et al.*, 2000b).

In contrast, BC7 both expresses this adhesin and binds CK13.

ATCC 25416 (American Type Culture Collection, Manassas, VA, USA) is a *cblA*-negative genomovar I strain and was used as a negative control.

Bacterial growth conditions. For DNA sequence determination and PFGE analyses, bacteria from frozen stock were recovered on Mueller–Hinton agar (Difco) after incubation at 35 °C for 24–48 h. For Southern and Western blot analyses, and for electron microscopy, bacteria from frozen stock were grown on brain-heart infusion agar (Becton Dickinson). For CK13-binding assay, bacteria were grown in tryptic soy broth (Difco), as described by Sajjan *et al.* (2000b).

***cblA* and upstream DNA sequence analysis.** By using *cblA*-specific PCR primers and reaction conditions previously described (Sajjan *et al.*, 1995) DNA fragments of the predicted size were amplified from the 11 study isolates (Table 1) as well as from the positive control strains BC7 and J2315. A second PCR assay employing forward primer 5'-GAGCTCGAATT-CGATATCGAGTGG-3' and reverse primer 5'-CTTGTCG-TTCGTGAAGATCTTCGTG-3' was designed to amplify DNA sequences immediately upstream of the *cblA* start codon. This assay was carried out in a 50 µl final volume that included 1.5 mM MgCl₂, 0.8 mM dNTPs, 0.4 mM of each primer, 100 ng template DNA and 1 U *Taq* DNA polymerase. PCR conditions were the same as for the *cblA* PCR except that an annealing temperature of 62 °C was used. Nucleotide sequences of *cblA* and the upstream segments were determined by using an ABI PRISM model 3700 DNA sequencer (Perkin-Elmer Applied Biosystems). Sequence analyses were performed using EditSeq and MegAlign software (DNASTar). The GenBank accession numbers for the complete *cblA* nucleotide sequences for the isolates listed in Table 1 are AF455151–AF455162.

PFGE. Isolate genotyping by using macrorestriction digest and PFGE was performed as previously described (Chen *et al.*, 2001). Strain identity was determined by using published criteria (Tenover *et al.*, 1995).

Southern blot analysis. Chromosomal DNA (10 µg) from each isolate was digested with *EcoRI* and subjected to electrophoresis in 0.8% agarose. The resulting DNA fragments were transferred to a nylon membrane, fixed by UV cross-linking and hybridized with a digoxigenin-labelled *cblA* probe as described by Sajjan *et al.* (1995). The bound probe was detected by using anti-digoxigenin antibody and a chemiluminescent substrate (CPD*, Roche Molecular Biochemicals).

Production of antibody to cable pilin protein. Cable pilin subunit protein was prepared from *B. cepacia* strain BC7 as described previously (Sajjan *et al.*, 1995). Two New Zealand White rabbits were injected subcutaneously with this preparation (10 µg), mixed with TiterMax classic adjuvant (Sigma-Aldrich), on days 1, 15, 30 and 45. Polyclonal antiserum was obtained 2 weeks after the last injection.

Reactivity to anti-cable pilus antibodies. Whole-cell extracts of *B. cepacia* isolates were prepared as described previously (Sajjan & Forstner, 1992). Bacterial surface proteins were isolated after incubating bacteria at 60 °C for 15 min as described by Cravioto *et al.* (1982). Whole-cell extracts or surface proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 3% BSA for 2 h at room temperature, incubated with anti-cable pilin antiserum (1:1000 diluted) overnight at 4 °C and washed. Bound antibody was detected using anti-rabbit

Table 1. *B. cepacia* complex isolates with *cblA* sequences

Isolate	Source*	State†	Genomovar	Identity with ET12 <i>cblA</i> (%)‡
AU0007	CF sputum	New York	III	100
AU0113	CF sputum	Texas	I	88.0
AU0143	CF sputum	Virginia	I	88.0
AU0666	CF sputum	North Carolina	I	88.4
AU0717	CF sputum	Georgia	I	88.2
AU1540	CF sputum	Georgia	I	88.4
AU1544	CF sputum	Georgia	I	88.2
AU1550	CF sputum	Georgia	I	87.8
AU1555	CF sputum	Virginia	I	88.2
AU1693	CF sputum	Georgia	I	88.2
BC20	Stream sediment	South Carolina	I	88.4

* CF sputum isolates obtained from 606 US patients infected with *B. cepacia* complex as previously reported (LiPuma *et al.*, 2001).

† US state in which the CF patient received care.

‡ Based on comparison with the J2315 *cblA* nucleotide sequence.

IgG conjugated with alkaline phosphatase (Bio-Rad), and a colour substrate (NBT-BCIP, Roche Molecular Biochemicals).

CK13 binding assay. Binding of *B. cepacia* to CK13 was determined as described previously (Sajjan *et al.*, 2000a). In brief, a cytokeratin-rich fraction, isolated from buccal epithelial cells as described by Franke *et al.* (1981), was subjected to electrophoresis and the proteins were transferred to a nitrocellulose membrane. Membranes were blocked with 1% gelatin for 1 h at room temperature, incubated with ³⁵S-labelled *B. cepacia* (1×10^9 c.f.u. ml⁻¹) for 90 min at 37 °C, washed to remove unbound bacteria and exposed to X-ray film.

Electron microscopy. Bacteria were grown on brain-heart infusion agar, lifted onto a Formvar-coated grid, and stained with 1% phosphotungstic acid for 30 s. Excess stain was removed by blotting with moist filter paper and the grid was allowed to air dry. Samples were examined by using a JEOL 1230 electron microscope equipped with a CCD camera.

RESULTS

Bacterial strains and *cblA* sequence analyses

The 11 isolates identified as containing *cblA* sequences are listed in Table 1. The 10 CF sputum isolates were recovered from 10 patients receiving care in six US states. The environmental isolate (BC20) was obtained from blackwater stream sediment from another US state (Wise *et al.*, 1995). Nine of the 10 CF isolates as well as the environmental isolate BC20 were confirmed as belonging to *B. cepacia* complex genomovar I based on *recA* PCR and RFLP analyses (Mahenthalingam *et al.*, 2000a). The remaining CF isolate (AU0007) belonged to genomovar III.

Although DNA fragments of the predicted size were amplified from all 11 isolates by *cblA*-specific PCR, only the product from the genomovar III isolate (AU0007) had 100% nucleotide sequence identity with the *cblA* amplified from the ET12 strains J2315 and BC7. The

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ET12:  GCGCCGAGCGCGAATAACGAGACCGGACGGGCATACCAT
              -35                               -10

GI:    GCGCCGAGCGCGAATAACGAGACCGGACGGGCATACCAT
              -35                               -10

ET12:  TGTTTCAACAACCAGAAGGATAACCCATG
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GI:    TGTTTCAACAACCAGAAGGATAACCCATG
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Fig. 1. Sequences of the 70 bp segment upstream of the *cblA* start codon (asterisks) from ET12 isolates (upper line) and genomovar I isolates (lower line). All 10 genomovar I isolates had identical DNA sequence in the 70 bp segment shown. Putative -10 and -35 sequences are underlined. Three nucleotide differences in the intervening segment are indicated by white letters.

cblA nucleotide sequences from the 10 genomovar I isolates showed high identity (range 98.2–100%) to each other, but lower identity (range 87.8–88.4%) to the ET12 *cblA* sequence. *cblA* sequences from three genomovar I isolates (AU1544, AU1555 and AU1693) shared 100% identity with each other.

To investigate whether differences in cable pili expression (see below) may result from upstream nucleotide sequence variations we amplified a 145 bp segment immediately upstream of the *cblA* start codon from all isolates. Again, the sequences from the ET12 isolates AU0007, J2315 and BC7 were identical to each other. The sequences from AU1544, AU1555 and AU1693 were also identical to each other and this sequence differed from the remaining seven genomovar I strains by a single nucleotide. The identity between the ET12 sequence and that of isolates AU1544, AU1555 and AU1693 was 96.6%; identity between the ET12 sequence and that of the remaining seven isolates was

A	MLKYVP	PIAAA	ALMSMS	AYAV	QKDI	TVTANV	DTTLE	MLSAD	GSAL	PTTMQM	QYLP	GTGLQA	60
B	MLKYVP	PIAAA	ALMSMS	AHAV	QKDI	TVTANV	DTTLE	MLSAD	GSAL	PTTMQM	QYLP	PGSGLQA	60
C	MLKYVP	PIAAA	ALMSMS	AHAV	QKDI	TVTANV	DTTLE	MLSAD	GSAL	PTTMQM	QYLP	PGSGLQA	60
D	MLKYVP	PIAAA	ALMSMS	AHAV	QKDI	TVTANV	DTTLE	MLSAD	GSAL	PTTMQM	QYLP	PGSGLQA	60
E	MLKYVP	PIAAA	ALMSMS	AHAV	QKDI	TVTANV	DTTLE	MLSAD	GSAL	PTTMQM	QYLP	PGSGLQA	60
A	AVVNT	KIFTN	DKAKD	LQIRL	ATAP	PALKNQT	SPGAA	EIPLS	VKLGE	TELTT	TAATL	KTAEL	120
B	AEVNT	KIFTN	DKAKD	LQIRL	AAEP	PALKNQT	SPGAK	EIPLA	VKLGT	TALST	TATTL	KASEI	120
C	AEVNT	KIFTN	DKAKD	LQIRL	AAEP	PALKNQT	SPGAK	EIPLA	VKLGT	TALST	TATTL	KASEI	120
D	AEVNT	KIFTN	DKAKD	LQIRL	AAEP	PALKNQT	SPGAK	EIPLA	VKLGT	TALST	TATTL	KASEI	120
E	AEVNT	KIFTN	DKAKD	LQIRL	AAEP	PALKNQT	SPGAK	EIPLA	VKLGT	TALST	TATTL	KASEI	120
A	FTGEL	AQGSN	VLALS	IQQK	VEAV	TASGSY	QGLVS	VIVTQ	SAASGS	167			
B	FTGEL	AQGSN	VLPLS	IEQKT	VGA	ITAAGSY	QGLVS	VIVTQ	SAASGS	167			
C	FTGEL	AQGSN	VLPLS	IEQKT	AGA	ITAAGSY	QGLVS	VIVTQ	SAASGS	167			
D	FTGEL	AQGSN	VLPLS	IEQKT	AGA	ITAAGSY	QGLVS	VIVTQ	SAASGS	167			
E	FTGEL	AQGSN	VLPLS	IEQKT	AGA	ITAAGSY	QGLVS	VIVTQ	SAASGS	167			

Fig. 2. Alignment of deduced amino acid sequences. Row A, major pilin subunit protein from ET12 isolates (J2315, BC7 and AU0007). Row B, *cbIA*-encoded peptide from isolate AU0113. Row C, *cbIA*-encoded peptides from isolates AU0143, AU0666, AU0717, AU1540 and BC20. Row D, *cbIA*-encoded peptide from isolates AU1544, AU1555 and AU1693. Row E, *cbIA*-encoded peptide from isolate AU1550. Amino acids differing from the ET12 pilin subunit protein are shaded.

Fig. 3. PFGE genotyping analysis of *cbIA*-positive isolates. Lanes 1 through 12, J2315, AU0007, AU0113, AU0143, AU0666, AU0717, AU1540, AU1544, AU1550, AU1555, AU1693, BC20, respectively. Profiles of J2315 and AU0007 are identical, as are those of AU1544, AU1555 and AU1693.

95.9%. Although the putative –10 and –35 sequences are identical among all ET12 and genomovar I isolates, the 16 bp intervening region contains 3 bp that differ between the ET12 and the genomovar I isolates (Fig. 1).

Predicted CblA amino acid sequence

Alignment of the deduced amino acid sequences is depicted in Fig. 2. The genomovar I strains showed 99% sequence identity to each other; they had between 85% and 86.2% sequence identity to the ET12 sequence. Differences in amino acid sequence between the genomovar I isolates and the ET12 sequence were detected mainly in the middle to the C-terminus of the predicted protein.

PFGE

Genotyping by PFGE demonstrated that the genomovar III isolate (AU0007) had a profile identical to strain J2315 and was thus indeed of the ET12 lineage (Fig. 3,

Fig. 4. Detection of *cbIA*-containing *EcoRI* fragments by Southern blot analysis. ATCC, ATCC 25416 (*cbIA*-negative). Only the relevant part of the blot is shown.

lanes 1 and 2). The DNA profiles of BC7 and J2315 have been shown in previous work to be the same as well (Mahenthalingam *et al.*, 2000b). Consistent with the *cbIA* sequence analyses (above), genomovar I isolates AU1544, AU1555 and AU1693 had the same profile (Fig. 3, lanes 8, 10 and 11) and are thus considered clonal; the remaining seven isolates had different genomic macrorestriction profiles, indicating that they represent different strain types.

Southern blot analysis

The mobility of the *cbIA*-containing *EcoRI* fragments from the 10 genomovar I strains differed from that of the ET12 strains (Fig. 4). The *cbIA*-containing fragments from all 10 genomovar I isolates were approximately 4 kbp in size, whereas that of the ET12 isolates was approximately 5 kbp.

Reactivity of anti-cable pilin antibody with *B. cepacia* isolates

Western blot analysis of bacterial whole-cell extracts and surface proteins using anti-cable pilin antibody was used to examine expression of the major pilin subunit. Cable pilin antibody reacted strongly with a 16 kDa

Fig. 5. Detection of major cable pilin protein by Western blot analysis. Whole-cell extracts from each *B. cepacia* isolate indicated were subjected to SDS-PAGE, and proteins were transferred to a nitrocellulose membrane. The membrane was incubated with anti-pilin antibody and the antibody was detected with a colorimetric reaction as detailed in the text. The first lane contains molecular mass standards. The 16 kDa cable pilin protein was detected only in the three genomovar III ET12 isolates, BC7, J2315 and AU0007. All genomovar I isolates are negative. ATCC, ATCC 25416.

protein in both whole-cell extracts (Fig. 5) and surface-protein preparations (data not shown) from the genomovar III ET12 isolates BC7, J2315 and AU0007. In contrast, neither whole-cell nor surface-protein extracts

from ATCC 25416, which lacks *cblA*, or any of the genomovar I strains reacted with this antibody under the conditions examined. This indicates either lack of expression of major cable pilin subunit protein or lack of antibody reactivity with an aberrant pilin subunit that may be expressed by these isolates.

Binding of *B. cepacia* to CK13

As previously demonstrated (Sajjan *et al.*, 2000a) cable-pili-expressing isolate BC7 exhibited strong binding to CK13. In contrast, neither AU0007 nor any of the genomovar I isolates bound to the membrane-immobilized CK13-enriched preparation (Fig. 6). Also as previously shown (Sajjan *et al.*, 2000b), J2315 did not bind to CK13.

Electron microscopy

Electron microscopy was used to investigate the surface expression of cable pili (Fig. 7). The positive control strain BC7 showed peritrichous pili tethered together to give the cable morphology described previously (Sajjan *et al.*, 1995) (Fig. 7a). None of several genomovar I strains (AU0143, AU1540, AU1544 and AU1555) demonstrated cable pili on their surfaces (Fig. 7b). Although isolate AU0007 expressed pili, it was hyperpililiated compared to BC7 and demonstrated numerous rigid pili that failed to entangle each other (Fig. 7c). Replicate

Fig. 6. Binding of *B. cepacia* isolates to CK13. A cytokeratin-rich fraction was isolated from buccal epithelial cells, transferred to a membrane, and the membrane was incubated with ³⁵S-labelled bacteria as described in the text. Only isolate BC7 showed binding to CK13, which has a molecular mass of 55 kDa. ATCC, ATCC 25416.

Fig. 7. Electron microscopy of negatively stained bacteria. Bacteria grown on brain-heart infusion agar were adsorbed on Formvar coated grids, stained with 1% phosphotungstic acid for 1 min and air-dried. The grids were observed under a JEOL 1200 EXII transmission electron microscope at 80 kV. Panels (a), (b) and (c) represent BC7, AU1544 and AU0007 respectively.

electron microscopic preparations gave reproducible results.

DISCUSSION

Although all species within the *B. cepacia* complex have been recovered from CF sputum culture, their distribution in CF patients is quite disproportionate (LiPuma *et al.*, 2001). In the US, genomovar III is recovered from approximately half of *B. cepacia*-complex-infected patients. In Canada, this species accounts for an even greater proportion of such patients (Speert *et al.*, 2002). This is due largely to the prevalence of the ET12 clonal lineage, a genomovar III strain that predominates among CF patients in Ontario and is believed to have spread between Canadian and UK patients during CF summer camps (Govan *et al.*, 1993; Johnson *et al.*, 1994).

The factors that account for the frequency of ET12 and other so-called 'epidemic' strains among CF patients are not clear. Few such strains have been characterized in detail (Mahenthiralingam *et al.*, 1997) and factors critical to inter-patient spread have yet to be defined. The *B. cepacia* epidemic strain marker (BCESM), a 1.4 kb sequence encoding an ORF with homology to transcriptional regulatory genes, is found in ET12 and other strains that infect multiple CF patients (Mahenthiralingam *et al.*, 1997). This sequence is generally absent from strains found only in single patients (i.e. for which there is no evidence of patient-to-patient spread). The role this element may have in contributing to inter-patient transmission is unknown.

Cable pili, another feature of the ET12 lineage, have been better characterized (Sajjan *et al.*, 1995; Sun *et al.*, 1995). These large intertwined peritrichous fibres, together with an associated adhesin, mediate bacterial adherence to respiratory epithelia via binding to CK13, a 55 kDa protein that has increased expression in CF (Sajjan *et al.*, 2000a). Recent work indicates that adherence mediated by cable pili also contributes to epithelial cell invasion and cytotoxicity during *B. cepacia* infection in CF (Sajjan *et al.*, 2002). Thus, expression of cable pili is of critical importance in the pathology of ET12, a strain accounting for significant infection in CF.

The identification of cable pili in ET12 initially raised hopes that screening of *B. cepacia* isolates for this phenotype (or, more specifically, for the presence of *cblA*) may allow identification of 'highly transmissible' lineages (Hearst & Elliott, 1995). Such screening could, in turn, enable CF centres to selectively apply stringent infection control measures, isolating only those patients harbouring an epidemic strain. Similar recommendations for stratification of infection control based on the presence or absence of *cblA* have been made more recently (Clode *et al.*, 2000).

However, such strategies are limited. Although cable pili mediate events important in the pathogenesis of infection by ET12, it is not yet clear whether expression of cable pili confers an enhanced capacity for inter-patient

transmission, per se. Other *B. cepacia* complex lineages implicated in inter-patient spread do not contain *cblA* sequences (Mahenthiralingam *et al.*, 1997). Indeed, PHDC, the genomovar III strain that predominates among CF patients in the mid-Atlantic region of the US, contains neither BCESM nor *cblA* (Chen *et al.*, 2001). The *B. multivorans* strain responsible for a hospital-associated outbreak among CF patients in the UK (Whiteford *et al.* 1995) similarly lacks BCESM and *cblA* (Mahenthiralingam, 2000b). Thus, neither *cblA* nor cable pili expression is a sensitive marker of *B. cepacia* strains that seem to possess an enhanced ability for spread in CF.

The results of the present study also indicate that *cblA* sequences are not specific for ET12. We identified several non-ET12 isolates positive for *cblA* by both dot-blot and PCR assays. Variant *cblA* genes with varying degrees of identity to the ET12 *cblA* gene were obtained from these isolates by PCR amplification using *cblA*-directed primers. Although we demonstrated pili expression by the ET12 isolates included in the study, none of the 10 *cblA*-containing non-ET12 isolates expressed pili under the conditions examined based on functional assays; this was confirmed by electron microscopy. The non-ET12 isolates represented several distinct strains by PFGE analysis, but interestingly all belonged to genomovar I, a species not commonly found in CF (LiPuma *et al.*, 2001). Furthermore, in all of these isolates the *cblA* sequences resided on an approximately 4 kbp *EcoRI* fragment, suggesting a common chromosomal location.

Others have similarly found *cblA* sequences among non-ET12 isolates, albeit at a low frequency. Sun *et al.* (1995) identified a single *cblA*-positive strain from among non-ET12 isolates recovered from over 100 CF patients. Similar to the isolates described in the present study, the *cblA* gene nucleotide sequence from this isolate had 88% identity with the ET12 *cblA* sequence. Although neither microscopic studies nor epithelial cell binding assays were performed, antibody against purified ET12 cable pili did not react with this isolate. Because description of this isolate preceded current knowledge of the taxonomy of the *B. cepacia* complex, the genomovar of this isolate is not known.

In a larger survey of 627 *B. cepacia* isolates representing 132 distinct strain types (defined by using randomly amplified polymorphic DNA typing) recovered from both CF and non-CF sources, Mahenthiralingam *et al.* (1997) detected DNA homologous to *cblA* in five non-ET12 isolates. Only one of these was from a CF patient; the remaining four were recovered from the environment. *cblA* sequence analysis and cable pili expression studies were not performed and again, genomovar analysis was not yet available.

More recently, Richardson *et al.* (2001) identified two non-ET12 strains, of unclear genomovar, containing *cblA* sequences from among 75 isolates of *Burkholderia* spp. Although functional assays of pili expression were not performed, the predicted amino acid sequences of these two strains had only 68% and 78% identity with

that of the ET12 pilin subunit protein. In contrast, a survey of 117 isolates from CF patients receiving care in 40 hospitals in the UK by Clode *et al.* (2000) demonstrated *cblA* sequences exclusively among ET12 lineage isolates; none of 76 non-ET12 isolates was *cblA* positive by PCR assay.

The frequency of *cblA* sequences in *B. cepacia* residing in the natural environment (or more specifically, among environmental genomovar I strains) is unknown. A more comprehensive survey of such isolates is needed to address this. Nevertheless, our findings suggest the possibility of inter-species transfer of potential virulence elements among *B. cepacia* complex species. Work is currently under way to assess this.

The reasons for the lack of cable pili expression in the *cblA*-positive genomovar I isolates identified in this study are not entirely clear. We found minor differences in the DNA upstream of the *cblA* start codon (specifically in the spacer region between the putative –10 and –35 sequences), and it is possible that this variation may affect transcription initiation. For example, substitution of AT by GC in this spacer region significantly increases the K_a of *E. coli* RNA polymerase (Auble *et al.*, 1987). Perhaps the replacement of C and G at positions –18 and –25, respectively, in the ET12 sequence by T in genomovar I isolates decreases the K_a of RNA polymerase, resulting in a reduced rate of transcription. It is also entirely possible that the differences noted in this promoter region do not account for the lack of pili expression by these isolates. Several other genes are probably involved in cable pili expression. Perhaps nucleotide differences in one or more of these, particularly those residing upstream of *cblA*, are responsible for the lack of pili expression in these isolates. Ongoing studies are assessing this possibility.

The reasons for the failure of AU0007 to express typical cable pili and the associated adhesin also require elucidation. This genomovar III CF isolate is clearly of the ET12 clonal lineage based on PFGE analysis. It also shares 100% nucleotide identity with the other ET12 isolates analysed (BC7 and J2315) in *cblA* as well as in the 145 bp segment immediately upstream of the *cblA* start codon. Nevertheless, it is hyperpilated with rigid-appearing pili and fails to express cable-pili-associated adhesin based on its lack of binding to CK13 (Sajjan *et al.*, 2000b); this is markedly different from other ET12 isolates examined to date. It is possible that the reasons for this lie elsewhere in the *B. cepacia cbl pil* operon; this is the subject of ongoing investigation.

In summary, although efforts to lessen the burden of strict infection control in CF are urgently needed, data from this study and those of others indicate that *cblA* lacks both the sensitivity and specificity to identify 'transmissible' strains. Until the factors contributing to the apparent proclivity of select *B. cepacia* complex strains to cause infection in CF are better defined, stratification of infection control measures will be problematic. In the interim, assays to assess cable pili expression (rather than the presence of *cblA* sequences)

may provide better specificity for identification of ET12 isolates. This will be particularly helpful in regions where tracking of ET12 is important in infection control surveillance.

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