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Cable (Cbl) Type II Pili of Cystic Fibrosis-Associated *Burkholderia* (*Pseudomonas*) *cepacia*: Nucleotide Sequence of the *cblA* Major Subunit Pilin Gene and Novel Morphology of the Assembled Appendage Fibers

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Previous studies have shown that appendage pili of *Burkholderia cepacia* strains isolated from patients with cystic fibrosis (CF) at The Hospital for Sick Children, Toronto, Canada, mediate adherence to mucus glycoproteins and also enhance adherence to epithelial cells. The specific pilin-associated adhesin molecule is a 22-kDa protein. In the present study we purified the major subunit pilin (17 kDa) and immunolocalized it to peritrichously arranged pili. On the basis of their novel morphological appearance as giant intertwined fibers, we refer to them as cable (Cbl) pili. Using an oligonucleotide probe corresponding to regions of the N-terminal amino acid sequence of the pilin subunit, we detected the encoding *cblA* gene in a chromosomal DNA library. Sequencing revealed this structural gene to be 555 bp in length, encoding a leader sequence of 19 amino acids, a cleavage site between the alanine at position 19 and the valine at position 20, and a mature pilin sequence of 165 amino acids. The calculated molecular mass is 17.3 kDa. Hydrophobic plus apolar amino acids account for 60% of the total residues. The pilin exhibits some similarities in its amino acid sequence to colonization factor antigen I and CS1 fimbriae of *Escherichia coli*. With the *cblA* gene used as a probe, hybridization assays of 59 independent isolates, including those from several geographically separated CF centers, plus environmental and clinical (non-CF) strains, gave positive results with all of the 15 CF-associated *B. cepacia* isolates from Toronto, plus a single strain from one other CF center (Jackson, Mississippi). The *cblA* gene is the first pilin subunit gene of *B. cepacia* to be identified.

Burkholderia cepacia is commonly found in soil and water and on plant surfaces. Ubiquitous occurrence is a major factor correlated with its phenotype as an opportunistic pathogen of humans. Over the past decade there has been a disturbing increase in the incidence of *B. cepacia* colonization of the lungs of patients with cystic fibrosis (CF). In about 30% of these patients, the clinical course is rapidly fatal over a few months to a year following acquisition of the organism (44, 45). Although the infection is believed to spread by nosocomial or direct patient-to-patient transmission (20), the factors permitting colonization are poorly understood. One factor of potential importance is the expression of surface pili. Together with outer membrane proteins, pili may play a role in the adhesion of *B. cepacia* to respiratory cells (15, 28, 31).

Pili of gram-negative bacteria consist of a major pilin subunit precursor, which is proteolytically cleaved to release a signal peptide during translocation across the cytoplasmic membrane and assembled by hydrophobic interactions into a filamentous polymer. The pilin structural subunits require interaction with several other gene products to permit the assembly of pili on the outer surface of the bacterium (12). The epithelial adherence function of pili is often mediated by a lectin-like protein (adhesin) which exists as a minor constituent associated with the mature pilus filament (12, 37).

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In previous studies of cystic fibrosis patients at The Hospital for Sick Children (Toronto, Canada), we found that isolates of *B. cepacia* were able to adhere to purified mucus glycoproteins and to buccal epithelial cells (29–31). By the use of mucin overlay assays during the purification of pili, we identified a specific 22-kDa mucin-binding adhesin. An antibody to the adhesin as well as purified mucin itself was shown by immunoelectron microscopy to be distributed sparsely along the length of large peritrichous pili of *B. cepacia*.

In the present report we describe the purification and immunolocalization of the major pilin subunit protein (molecular mass, 17 kDa) and its assembly into a novel, macromolecular structure. We also report the DNA sequence of the encoding chromosomal gene and its deduced amino acid sequence. Epidemiological analyses of *B. cepacia* isolates from several CF centers, plus environmental and clinical non-CF sources, are presented.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Table 1 provides details as to the sources and relevant biological properties of both the clinical (CF and non-CF) and environmental *B. cepacia* isolates and the reference strains used in the study. Bacteria were stored at −70°C in 5% trisodium citrate in 40% glycerol and grown under aerobic conditions on brain heart infusion agar for 24 to 48 h prior to use.

Purification of a major pilin protein from *B. cepacia*. Bacteria (CF isolate *B. cepacia* BC7) were scraped from agar plates and suspended in 0.01 M Tris HCl buffer (pH 7.2) containing 0.15 M NaCl (TBS), and pili were isolated by combining the methods of Paranchych et al. (23) and Dodd and Eisenstein (6). After the second (20%) ammonium sulfate precipitation (34), the resulting pellet (designated semipure pili) was further processed by gradient centrifugation on a urea-sucrose cushion (6). The final pellet was designated purified pilin protein.

TABLE 1. Bacterial strains

Strain(s)	Source and/or relevant biological information (reference[s])	Provider
B. cepacia BC5, -7, -18, -19, -21, -23, -24, -25, -29, -36, -37, -38, -42, -45, -61	The Hospital for Sick Children, Toronto, Canada; CF patients (29)	M. Karmali, Department of Microbiology
P. maltophilia PM11, PM12	The Hospital for Sick Children, Toronto, Canada; CF patients	M. Karmali, Department of Microbiology
B. cepacia reference strains 13945, 17759, 17765, 25416, 25609, 27515, 20352, 39277	13945, human endocarditis; 17759, forest soil; 17765, human urinary tract; 25416, onion (American Type Culture Collection type strain); 25609, human bronchial lavage sample; 27515, human tibial fracture; 29352, oil field soil; 39277, cornfield soil	American Type Culture Collection
B. cepacia 566, 792, 1823, 1845, 1903, 1910, 1948, 1963, 2028, 2034, 2047, 2181, 2211, 2214, 2297, 2308, 21050	Cystic Fibrosis Center, University of North Carolina Hospitals, Chapel Hill; CF patients	P. Gilligan, Department of Microbiology, University of North Carolina, Chapel Hill
B. cepacia 2323	University of Mississippi CF Center, Jackson; CF patient	P. Gilligan
B. cepacia 521, 523, 524, 544, 525	CF Center, Children's Hospital and St. Christopher's Hospital, Philadelphia, Pa.; CF patients	T. Stull and P. Gilligan
B. cepacia BC103, BC109, BCE^{2}_{13}	CF Center, St. Christopher's Hospital, Philadelphia, Pa. (15), and University of Washington, Seattle	P. Gilligan and A. Smith, Department of Infectious Diseases, University of Washington, Seattle
B. cepacia SBC27, SBC29	Cystic Fibrosis Clinic, University of Edinburgh, Edinburgh, Scotland; CF patients	J. S. Govan, Department of Microbiology
P. aeruginosa PAK, PAO1	Janeway CF Clinic, St. John's, Newfoundland, Canada (23)	R. Irvin, Department of Microbiology, University of Alberta, Edmonton, Canada
P. aeruginosa PA103	Encodes *p11* adhesin operon (32, 40); CF patient	S. Lory, Department of Microbiology, University of Washington
P. maltophilia PM11	Cystic Fibrosis Center, University of North Carolina Hospitals, Chapel Hill; CF patient	P. Gilligan, Clinical Microbiology Laboratory
E. coli H10407	Serotype O78:H11; encodes CFA/I	American Type Culture Collection
E. coli J96	Human pyelonephritis isolate, encoding *pap*/*prs* and *pil* adhesin operons; Stanford University Medical School, Stanford, Calif.	S. Normak, Department of Microbiology, Umea University

From the initial bacteria grown on 20 agar plates (150 by 15 mm), the total yield of purified pilin protein was 4.5 mg.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). At each stage of purification, samples were subjected to electrophoresis under reducing conditions (16) on 12.5% or 14% polyacrylamide minigels and stained with Coomassie blue. Western blot (immunoblot) immunoassays were carried out as described previously (30).

Compositional analyses. Total protein was assayed by the bicinchoninic acid protein assay (Pierce Chemical Co., Rockford, Ill.). Amino acid analyses were performed with the Picotag high-performance liquid chromatography system (10). For N-terminal amino acid sequences, immobilized proteins were analyzed by the Edman degradation procedure using a Porton gas-phase microsequencer, model 2090. To obtain internal peptides, the immobilized pilin protein (50 μg) was subjected to in situ CNBr cleavage (10 mg of CNBr in 70% formic acid) by using the Probe-design peptide separation system of Promega (Madison, Wis.). Peptide fragments were separated by SDS-PAGE (35), transferred to Problott membranes (Promega), stained with Coomassie blue, and subjected to N-terminal sequence analysis (Biotechnology Service Facility, The Hospital for Sick Children, Toronto, Canada).

Production of an antipilin antibody. The 17-kDa pilin protein (20 μg) was cut

and electroeluted from polyacrylamide gels, and together with Freund's complete adjuvant, it was injected into the peritoneal cavities of CD1 mice (Charles River Laboratories, Quebec, Canada) on days 0, 7, 14, and 21. Ascitic fluid was processed for antibody development (9) and evaluated by slot blot enzyme-linked immunoassay, by using the pilin protein (or bovine serum albumin [BSA] as a control) as an antigen and a second goat anti-mouse immunoglobulin G conjugated to alkaline phosphatase (Bio-Rad, Richmond, Calif.). Color was developed with the Promega NBT-BC1P substrate system. Serum taken from mice before injection of the antigen was used as a background control.

Electron microscopy. Immunoelectron microscopy was performed on isolate *B. cepacia* BC7 (and *Pseudomonas maltophilia* PM11 as a control) as described previously (30) by using the antipilin antibody and a goat anti-mouse antibody–colloidal gold (10-nm-diameter particles) conjugate as a probe.

For fine structural analysis, the 51 *B. cepacia* isolates cited in Table 1 were characterized by using minimal-beam, high-resolution transmission electron microscopy as described in the companion article (7a).

Preparation of oligonucleotide probes. Chromosomal DNA of *B. cepacia* BC7 was prepared (1) and amplified by PCR with a degenerate sense primer (5′-GTICAA/GAAA/GGATATIACIGTIACIGCIAAI-3′) corresponding to residues 1 to 10 and an antisense primer (5′-ITAITGCATT/CTGCATIGTIGT-3′) corresponding to amino acids 27 to 33 of the 17-kDa pilin protein (I is deoxyinosine). PCR mixtures (100 μl) contained primers (1 μM each), genomic DNA (500 ng), deoxynucleoside triphosphates (200 μM each), *Taq* polymerase (2.5 U; Perkin-Elmer Cetus, Norwalk, Conn.), $MgCl_2$ (2.5 mM), and 0.01 M Tris HCl (pH 8.3) containing 0.05 M KCl. Amplification was for 35 cycles, each consisting of 1 min at 94°C, 1 min at 50°C, and 1 min at 72°C. The product was electrophoresed on 1% agarose gels, stained with ethidium bromide, and visualized under UV light (33). The PCR product (99 bp) was then ligated into the pCR 1000 plasmid vector by using the TA cloning system (Invitrogen) and transformed into *Escherichia coli* INVaF′ (TA Oneshot competent cells supplied with the kit), and the cells were grown on Luria-Bertani agar containing kanamycin (50 μg/ml). Plasmid DNA from recombinants was isolated (11), and the inserts were sequenced in both directions (34) by using the vector M13 reverse and −20 forward primers with T7 DNA polymerase (Pharmacia). A specific 30-nucleotide (nt) oligomer was synthesized to correspond to amino acids 11 to 20 of the purified pilin protein (5′-GTCGACACGACGCTCGAAATGCTGTCGGCG-3′) and 3′ end labelled with digoxigenin-11–dUTP by using terminal transferase and the Genius 6 oligonucleotide tailing kit (Boehringer Mannheim Biochemicals, Indianapolis, Ind.).

Cloning of the *cblA* pilin structural gene. Chromosomal DNA (10 μg) of *B. cepacia* BC7 was digested with restriction endonucleases *Eco*RI and *Pst*I (Pharmacia LKB Biotechnology, Uppsala, Sweden), the digests were electrophoresed through 0.8% agarose gels, and the products were transferred by Southern blotting to nitrocellulose (Schleicher and Schuell, Inc.) or nylon membranes (Boehringer Mannheim) by capillary action (38). Hybridization was carried out at 65°C with the 30-nt probe, and the bound probe was detected colorimetrically by using the Genius system (Boehringer Mannheim Biochemicals). Probe-positive fragments were cut, electroeluted from the gels, and ligated into pUC 18; plasmids were transformed into *E. coli* DH5α competent cells (Gibco-Bethesda Research Laboratories, Bethesda, Md.); and cells were grown on Luria-Bertani agar containing 100 μg of ampicillin per ml. Transformants were transferred to BAS-85 nitrocellulose filters (Schleicher and Schuell) and processed for hybridization (33) with the digoxigenin-labelled 30-nt probe. Plasmid DNA was isolated from positive clones and digested with *Eco*RI or *Pst*I, and inserts were verified by Southern blot hybridization analyses. Sequencing of inserts was carried out in both directions with a T7 sequencing kit (Pharmacia) by using M13 and T7/T3a universal primers, the 30-nt pilin-specific oligonucleotide (as an internal primer), and overlapping synthetic primers.

Prepilin sequence probe. *B. cepacia* (isolate BC7) genomic DNA (500 ng) was amplified by PCR with primers complementary to sequences upstream of the translation start site and downstream of the termination site of the prepilin gene. The sense primer, 5′-CCAAAGGACTAACCCA-3′, corresponded to nt −57 to −72, and the antisense primer, 5′-ACGCGATGTCCATCACA-3′, corresponded to nt 583 to 599 (see Results). Amplification cycles were 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. The PCR product (664 bp) was cloned into pCR 1000, and its sequence was verified. The fragment was excised from the vector with *Kpn*I and *Eco*RI enzymes, and the 722-bp product containing the pilin gene was labelled by random priming either with digoxigenin-dUTP or with [α-^{32}P]dCTP (Dupont, NEN Research Products, Boston, Mass.) (7).

DNA hybridization assays. A survey of the strains listed in Table 1 was carried out by dot blot hybridization (5) with the ^{32}P-labelled pilin probe, followed by autoradiographic detection of signals. For Southern blot hybridization assays, chromosomal DNA was isolated from *B. cepacia* strains (39, 46), digested with *Eco*RI, electrophoresed through a 0.8% agarose slab gel, and transferred to a Zeta-probe membrane (Bio-Rad Laboratories). Hybridization analyses (2, 39) were performed with the same pilin probe.

Computer analyses. DNA and protein sequence homology searches were carried out on a VAX computer system with the MBIR software package (26) by using the GenBank nucleic acid database (release 71) and the protein sequence database of the National Biomedical Research Foundation Protein Identification Resource (PIR release 32). The QFASTA.SH algorithm program (19) was used for alignments of pilin protein sequences to maximize homologies.

Nucleotide sequence accession number. The nucleotide sequence of the *cblA* major subunit pilin gene has been submitted to the GenBank data library under accession no. U10244.

RESULTS

Morphology of pili. As described in detail in the companion article (7a), the electron microscopic appearance of *B. cepacia* differed according to the specific procedure used for transferring bacteria to the grids. With low shear levels (method a), 43 CF-associated isolates from six geographically separated CF centers (Table 1) were found to possess an intertangled meshwork of fine appendage filaments. We refer to these structures as "mesh" (Msh) type I *B. cepacia* pili (Fig. 1 in the accompanying article [7a]). The biochemical features of these pili are currently under study. Preliminary results indicate that the Msh pilin subunit is a 37-kDa protein (48). In addition, however, 14 of the 15 Toronto CF isolates plus the single Mississippi CF isolate possessed fibers having much greater diameters and lengths, which were somewhat hidden within the mesh (see Fig. 1B in the accompanying article [7a]). When isolates were applied to the grid by the spray drop technique (method b), the Msh pili were sheared off and the larger fibers were revealed as outwardly projecting, peritrichous pili (Fig. 1). Because of their cable-like appearance, we refer to them as cable (Cbl) type $II_{B.\ cepacia}$ pili. They typically radiated for enormous distances (2 to 4 μm) from the bacterial surface, often appearing to tether adjacent groups of bacterial cells into mat-like configurations. *B. cepacia* isolates from other CF centers also expressed a second category of pili in addition to Msh pili (see reference 7a), but none displayed the giant Cbl pili associated with the Toronto isolates and the single isolate from the Mississippi CF center.

Isolation, immunolocalization, and N-terminal sequence of the Cbl type II pilin subunit. Pili were isolated from the Toronto CF isolate *B. cepacia* BC7, one of several isolates previously shown to bind to both mucins and epithelial cells by means of the 22-kDa pilin adhesin (29–31). The initial bacterial homogenate contained over 30 protein bands (30), but after a second ammonium sulfate precipitation to yield semipure pili, there was one major protein at 17 kDa and another minor band was observed at approximately 45 kDa (Fig. 2). The 22-kDa adhesin was also present at this stage of the purification, but it was detected only with Western blots for which a specific antiadhesin antibody was used (30). After centrifugation of semipure pili on a urea-sucrose-Tris cushion, the pellet was subjected to SDS-PAGE, and it produced a single Coomassie blue-stained band at 17 kDa. This band was not recognized in Western blots by the antiadhesin antibody. The band was cut and electroeluted from the gel, and a polyclonal antibody to it was developed and used for immunoelectron microscopy. The optimal conditions for visualizing the antibody included bacterial transfer to grids by method b, which removed most of the mesh (Msh) pili. The 17-kDa protein was immunolocalized over the length of the large projecting Cbl type II pili (Fig. 3). Preimmune serum produced no reaction, and a negative control isolate of *P. maltophilia* (PM11) failed to react with the antipilin antibody.

The 17-kDa pilin protein yielded a single N-terminal peptide sequence consisting of 42 residues. In situ digestion of the protein with CNBr yielded one peptide of approximately 14 kDa. A 10-amino-acid segment of its 27-residue N-terminal sequence overlapped with residues 32 to 42 of the intact pilin protein, such that the CNBr fragment represented amino acids 32 to 59 of the pilin protein.

Cloning and sequencing of the *cblA* pilin gene. A 30-nt probe

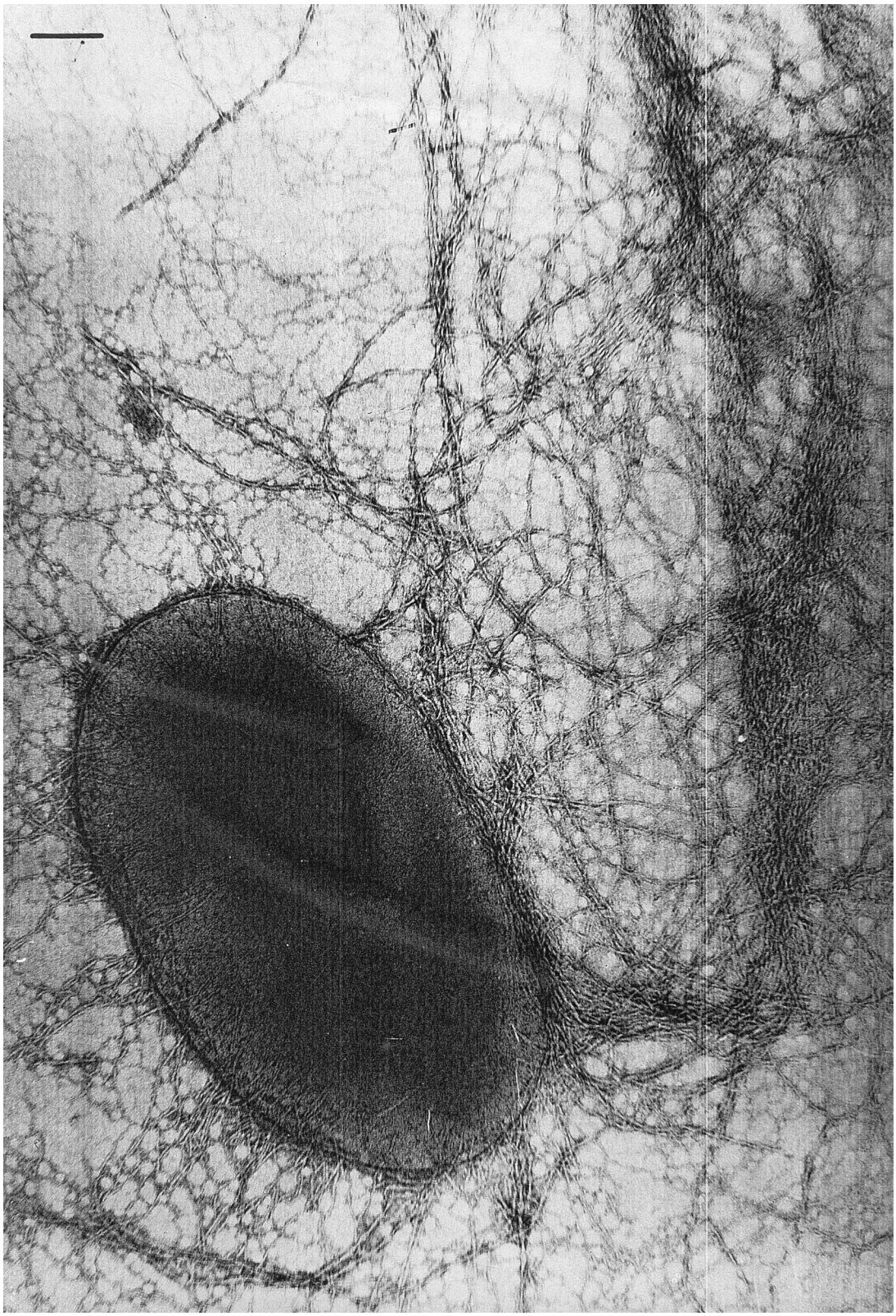


FIG. 1. High-resolution transmission electron micrograph of *B. cepacia* expressing Cbl type II pili. Bar, 0.1 μm.

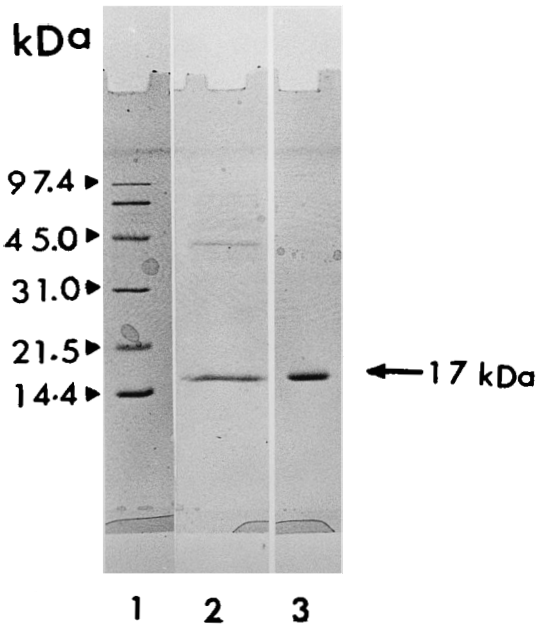


FIG. 2. SDS-PAGE separation of *B. cepacia* Cbl pilin protein. Samples (12 μg) were applied under reducing conditions to 14% polyacrylamide gels, and protein bands were detected by staining with Coomassie blue. Lane 1, molecular mass standards; lane 2, semipure pili; lane 3, purified pilin protein.

corresponding to amino acids 11 to 20 of the pilin subunit was used in Southern blot hybridization analyses of *B. cepacia* BC7 genomic DNA. Two positive fragments (0.7 kb from *Pst*I digests and 6 kb from *Eco*RI digests) were used to generate partial libraries, which were screened with the digoxigenin-labelled 30-nt oligomer. From 328 and 141 recombinants, respectively, one positive clone containing the *Pst*I-generated fragment (designated 7_1) as well as a clone containing the *Eco*RI fragment (designated 5_2) was selected for sequencing. Near its 5′ end, clone 5_2 (ca. 6-kb insert) contained the entire open reading frame (555 bp) of the pilin structural gene (nt −57 to +499; Fig. 4).

Sequence of the *cblA* gene and the encoded Cbl type II$_{B.\ cepacia}$ pilin protein. Beginning at nt 202 from the 5′ end of clone 5_2 (a position which has been assigned number 1 in Fig. 4), the deduced amino acid sequence matched that determined biochemically for residues 1 to 59. Three potential initiation codons were determined to be present at positions −15, −21, and −57, but the last was selected because it was preceded by a consensus Shine-Dalgarno sequence, AGGA (36), at positions −65 to −69. The pilin protein contains a deduced leader sequence of 19 amino acids, a cleavage site (arrow) between alanine −1 and valine +1, and a mature pilin protein of 165 amino acids. The leader sequence is typical of many other prokaryotic signal sequences in containing a charged residue within the first 5 amino acids (lysine at position 3), a long hydrophobic core, and a cleavage site following the uncharged

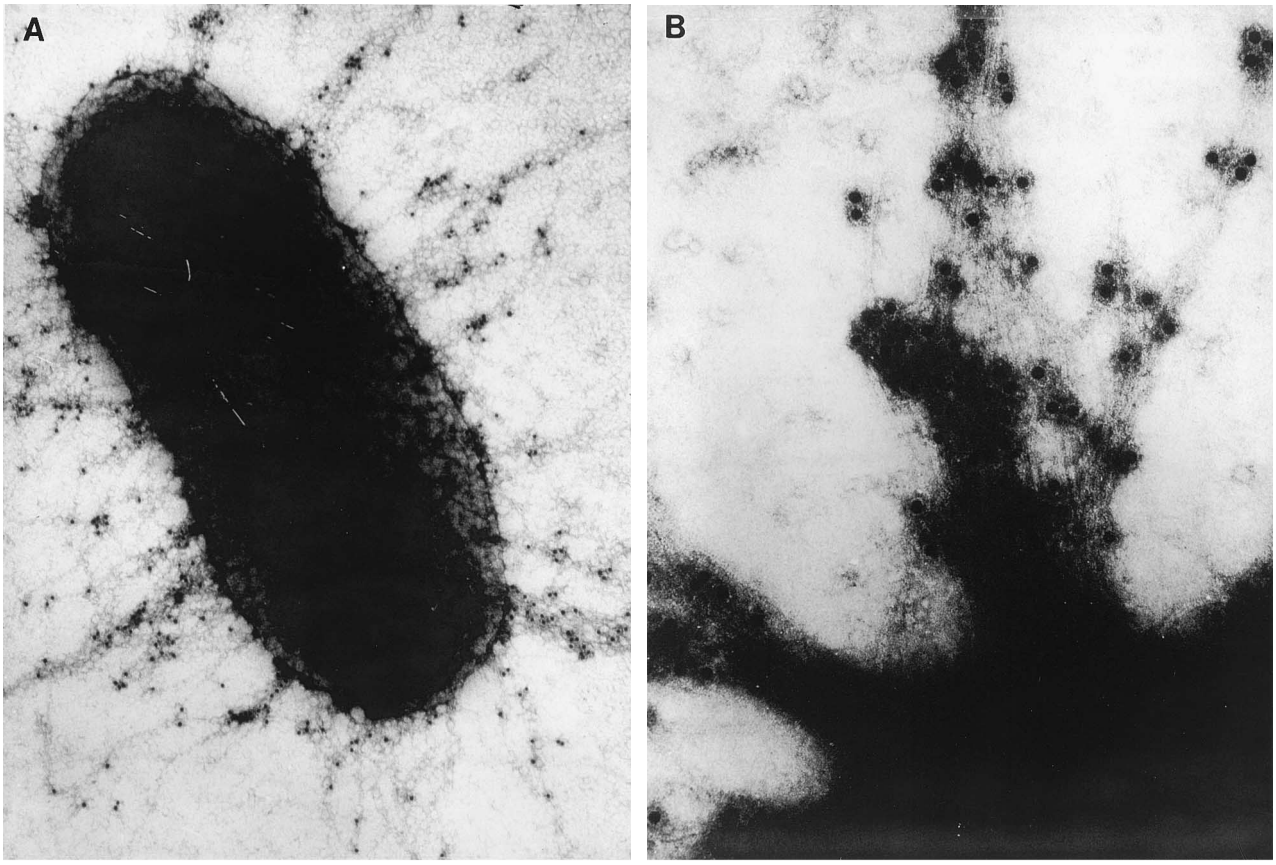


FIG. 3. Immunogold localization of the Cbl pilin protein. *B. cepacia* BC7 was adsorbed onto copper-coated grids, fixed with 1% glutaraldehyde, blocked with 3% BSA, and incubated with the antibody (1:100 dilution) to the 17-kDa pilin protein. Grids were washed and incubated with goat anti-mouse immunoglobulin G conjugated to colloidal gold (10-nm-diameter particles), counterstained with 0.05% uranyl acetate, and examined under a Philips 400 T electron microscope. Magnification, ×25,116 (A) or ×103,500 (B).

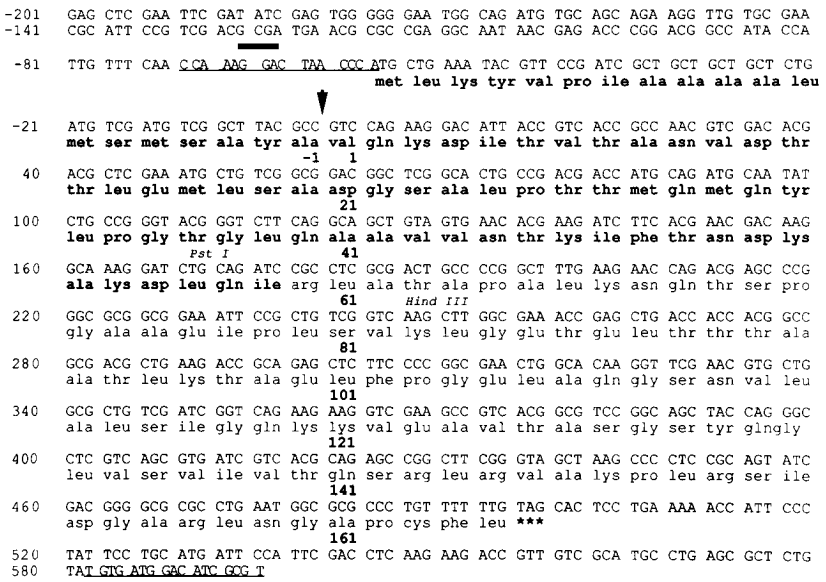


FIG. 4. Nucleotide sequence and deduced amino acid sequence of the *B. cepacia cblA* pilin gene. The numbering of nucleotides (left margin) and amino acids (central) is referenced to the valine at the N terminus of the mature pilin (vertical arrow). The deduced leader peptide spans residues −19 to −1. The Shine-Dalgarno AGGA sequence is overlined by a horizontal bar, and the stop codon at the 3′ end is indicated by three asterisks. Deduced amino acids 1 to 59 (boldface) were also confirmed biochemically. Internal *Pst*I and *Hin*dIII sites are indicated. The prepilin gene spans a total of 555 nt (encoding 184 amino acids). The underlined nucleotide sequences (−57 to −72 and 582 to 598) were used to construct primers for PCR generation of a full-length pilin gene probe.

alanine (47). The mature pilin was calculated to have a molecular mass of 17.3 kDa, which agrees with the size estimated by SDS-PAGE. There was a close correspondence of the measured and deduced amino acid compositions of the mature pilin (data not presented), which differ from the composition of the 22-kDa adhesin (30). The hydrophobic nature of the pilin was striking, as judged from the high content (approximately 60%) of hydrophobic plus apolar residues. This finding is consistent with the well-recognized role of hydrophobic interactions in stabilizing pilin subunit polymerization (14).

A computer search revealed no nucleic acid homology of this pilin with any other sequence reported in the GenBank library, including those encoding pili of *E. coli* and *Pseudomonas aeruginosa*. The calculated GC content of the *B. cepacia* pilin gene was 62%, which is intermediate between the GC content of genomic DNA of *E. coli* (52% mean value) and that of *B. cepacia* genomic DNA (67%) (22) but is much higher than the GC contents of the *E. coli* genes for CS1 (45%) or colonization factor antigen I (CFA/I) (40%) pilin subunits (14, 27). These comparisons were made because many regions of amino acid sequence similarity among the *B. cepacia* Cbl pilin subunit and the CS1 and CFA/I fimbrial subunits of *E. coli* were recognized (Fig. 5). The Cbl pilin exhibited 35 and 30% overall amino acid identity with CS1 and CFA/I fimbriae, respectively, but allowing for conservative replacements and three gaps to optimize alignments, the similarities increased to 74.4 and 71.7%, respectively. It has been reported that there is no cross-reactivity between antisera for CS1 and CFA/I fimbriae (27), and in the present study we found no reactivity of the antibody to the Cbl pilin with CFA/I-containing *E. coli* O78:H11 (data not presented).

Hybridization-based epidemiological assays for the *cblA* pilin gene among environmental, CF-associated, and non-CF clinical isolates. By using dot blot hybridization, a survey for *cblA* was carried out with the bacterial strains listed in Table 1. Figure 6 reveals that all 15 of the Toronto *B. cepacia* isolates carry the *cblA* structural gene (rows 1 and 2), as well as one other isolate (no. 2323; Jackson, Mississippi) (row 2, column f). Other CF-associated strains, plus diverse environmental and clinical *B. cepacia* isolates and non-*B. cepacia* controls (Table 1), gave no hybridization signal.

Southern blot hybridization assays using a ^{32}P-labelled *cblA* gene probe were performed on *Eco*RI digests of DNA purified from isolates by CsCl equilibrium density gradient centrifugation (46). Representative results are displayed in Fig. 7. All 15 of the Toronto isolates (of which 4 are shown) exhibited a single probe-positive band at ca. 6 kbp. Among the other isolates (Table 1), only the *B. cepacia* strain (no. 2323) from Jackson, Miss., was positive for *cblA*, in this case showing two bands of slightly greater mobility.

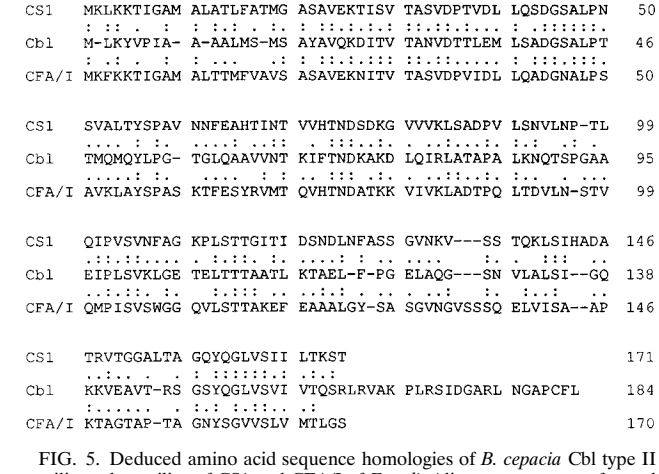
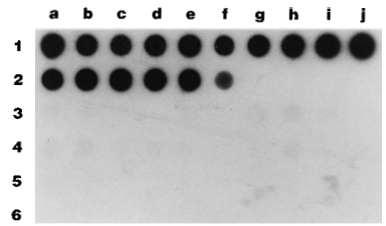


FIG. 5. Deduced amino acid sequence homologies of *B. cepacia* Cbl type II prepilin and prepilins of CS1 and CFA/I of *E. coli*. Alignments were performed by use of the QFASTA.SH algorithm. Gaps (–) were introduced to optimize alignments. Identical residues are indicated by double dots, and conservative replacements are indicated by single dots.



	a	b	c	d	e	f	g	h	i	j
1	5	7	18	19	21	23	24	25	29	36
2	37	38	42	45	61	2323	13945	17759	17765	25609
3	29352	27515	25416	39277	2028	1910	2047	2214	1823	566
4	21050	1963	792	1948	2034	2297	2308	2211	1845	1903
5	2181	S2	544	521	523	524	525	SBC 27	SBC 29	PA 103
6	XM 11	EC J96								

FIG. 6. Dot blot epidemiological analysis for the *cblA* gene. The grid in the lower portion of the figure indicates the corresponding order of particular strains analyzed as shown immediately above by hybridization with the ^{32}P-labelled *cblA* gene. Table 1 provides relevant information for each strain.

To determine whether *cblA* might also be plasmid encoded, plasmid and chromosomal DNAs were separated electrophoretically (3, 17), and bands were transferred to nitrocellulose and hybridized with the ^{32}P-labelled *cblA* probe. In the case of none of the 16 isolates for which *cblA* sequences had been detected by dot blot or Southern hybridization analyses was a positive signal obtained for the naturally occurring plasmids carried by the strain (data not presented).

DISCUSSION

The results described in this report provide the first identification of a pilin subunit structural gene (designated *cblA*) of *B. cepacia*. Its sequence and the morphological structure into which the translated *cblA* pilin subunit is assembled appear novel in a number of ways.

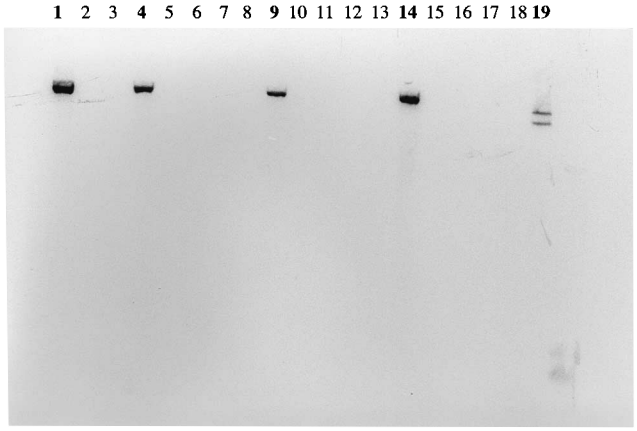


FIG. 7. Typical Southern blot hybridization results of *Eco*RI digests of *B. cepacia* chromosomal DNA probed with the ^{32}P-labelled *cblA* gene. Lanes 1, 4, 9, 14, and 19 show a positive signal at ca. 6 kbp for Toronto CF isolates BC5, BC7, BC45, and BC61 and Mississippi CF isolate 2323, respectively; lanes 2 and 3 contain Philadelphia CF isolates 544 and 521; and lanes 5 to 8 and 10 to 13 contain eight independent American Type Culture Collection isolates listed in Table 1.

(i) *cblA* gene sequence and origin. The pilin peptide seems to be somewhat more characteristic of *E. coli* pilins than those of *Pseudomonas* species (13, 25, 47). For example, the peptide contains a typical long hydrophobic leader sequence terminating in an alanyl valine cleavage site and considerable sequence homology in many regions with the CFA/I (14) and CS1 (27) fimbriae of *E. coli*. In addition, the GC content of the *B. cepacia* pilin is intermediate between that of genomic DNA of *E. coli* and that of *B. cepacia* genomic DNA (22). These findings raise the possibility that the pilin gene was acquired by horizontal transfer from another bacterial species. Apart from the Toronto CF isolates and one other isolate (Mississippi strain 2323), none of the surveyed *B. cepacia* isolates from diverse sources carried the Cbl type II pilin subunit gene. This picture contrasts dramatically with the prevalence of the *pilA* pilin gene of *P. aeruginosa*. Despite the polymorphism of this latter gene (4), strains from exceedingly diverse origins typically carry enough sequence homology to be detected by a full-length *pilA* gene probe (32, 41).

(ii) Lack of homology between the *B. cepacia cblA* pilin subunit gene and the *P. aeruginosa pilA* pilin subunit gene. With the use of a pilin gene probe of *P. aeruginosa* PAK (24, 40) Kuehn et al. (15) reported positive hybridization with *B. cepacia* 103 (formerly PC 103), an isolate obtained originally from a Philadelphia CF patient. In our own studies, however, an identical PAK pilin gene probe repeatedly failed via Southern hybridization to detect homologous sequences with any of the *B. cepacia* isolates listed in Table 1, including the previously reported probe-positive strain BC103 (data not shown). In contrast, the control strain (PAK) from which the PAK pilin gene was cloned was positive, as were approximately 80 other independently isolated *P. aeruginosa* strains in our collection (41).

B. cepacia 103 (and associated strains BC109 and BCE$^{2}_{13}$) (15) also failed to hybridize with the *cblA* gene even under low-stringency conditions. Negative results were also obtained for the piliated *P. aeruginosa* CF strains PAK and PAO1. Our studies therefore fail to support the suggestion (15) that pili of the two species share regions of homology.

(iii) Copy number of *cblA*. Recent DNA-based analyses indicate that the 15 Toronto CF-associated *B. cepacia* strains belong to a set of very closely related subribotypes (42) and produce almost identical (<10% variation) pulsed-field gel electrophoresis-resolved chromosomal profiles (43). Given the apparent clonality of these isolates, it is not surprising that all were found to carry *cblA* in the same, single-copy-number configuration (Fig. 7). In contrast, the Jackson, Miss., CF center strain 2323, a genetically distantly related isolate of significantly variant ribotype and pulsed-field gel electrophoresis type (7b), produced two hybridization bands. Current sequencing of *cblA* from strain 2323 should reveal whether there exists an *Eco*RI site that could account for the two hybridization signals or whether two separate copies of *cblA* exist within the chromosome of this isolate.

(iv) *cblA* as a potential epidemiologic marker. Unlike the *cblA*-positive strains of the Toronto CF center, the *cblA*-negative strains at other CF centers, e.g., the University of North Carolina center, are only distantly related to each other, as judged from DNA analyses (39). This observation raises the question as to whether *cblA* could be a marker of a highly infectious, epidemically cross-spreading lineage of *B. cepacia*. To address this question, it will be necessary to determine if *cblA* correlates with other well-defined epidemic strains at geographically distant CF centers. It would thus be relevant to determine whether isolates other than no. 2323 from the Jackson, Miss., CF center carry *cblA* and, if so, to determine their genetic relatedness. Likewise, because the two *cblA*-negative

Edinburgh CF isolates used in this study do not belong to the highly transmissible, virulent United Kingdom strain cluster described by Govan et al. (8), it will now be important to examine the latter group.

(v) Structure and function of Cbl pili. The intertwined quaternary arrangement of cable (Cbl) pili does not resemble the arrangement of pili reported for any other bacterial species, and it leads us to hypothesize that it could reflect an evolutionary selection associated with colonization of the lung chronically damaged by CF. Several features make this hypothesis attractive. For example, isolates expressing Cbl type II pili have been shown in earlier studies to adhere to mucins (29), and in CF airways, mucins typically form an abnormally thick blanket over the epithelium. Thus, the mucus biofilm may provide a favorable niche for colonization. Secondly, binding both to mucins and to the 55-kDa epithelial cell receptor (31) is mediated by the minor pilus component of 22 kDa. In contrast to the *pap*/*prs* and *pil* operon-encoded pili of *E. coli* (18, 21), the 22-kDa adhesin is not located solely at the appendage tip but is dispersed along the length of the cabled fiber (30). This distribution would be expected to maximize pilus interactions with mucus networks as well as with receptors on intact or sloughed epithelial cells. Thirdly, by braiding or cabling together, the long individual fibers of the Cbl pili may be stabilized against rupture by the shearing forces of ciliary beating and chronic coughing. Lastly, the tendency of the Cbl pili to intertangle with similar cables from neighboring bacteria would be expected to enhance the survival of bacterial colonies.

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ADDENDUM IN PROOF

After acceptance of this paper, ongoing sequencing of the *cblA* gene carried by pulsed-field gel electrophoresis of ribotype-identical *B. cepacia* isolates cultured from four CF patients has revealed in all cases the presence of an extra guanine at position 427 (see Fig. 4). This causes a frameshift such that the stop codon TAA occurs at nt 422 to 444. Thus, the correct sequence at the 3′ end of the gene is GCG GCT TCG GGT AGC TAA (nt 427 to 444), with the corresponding amino acid sequence being Ala-Ala-Ser-Gly-Ser (amino acids 143 to 147).

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