Identification and molecular analysis of cable pilus biosynthesis genes in *Burkholderia cepacia*

Umadevi S. Sajjan,¹ Hong Xie,¹ Matthew D. Lefebre,² Miguel A. Valvano² and Janet F. Forstner¹

¹Division of Structural Biology and Biochemistry, The Hospital for Sick Children, Toronto, Ontario, Canada M5G 1X8
²Department of Microbiology and Immunology, The University of Western Ontario, London, Ontario, Canada N6A 5C1

*Burkholderia cepacia* is an opportunistic respiratory pathogen in cystic fibrosis patients. One highly transmissible and virulent clone belonging to genovar IIIa expresses pili with unique cable morphology, which enable the bacterium to bind cytokeratin 13 in epithelial cells. The *cblA* gene, encoding the major pilin subunit, is often used as a DNA marker to identify potentially virulent isolates. The authors have now cloned and sequenced four additional genes, *cblB*, *cblC*, *cblD* and *cblS*, in the pilus gene cluster. This work shows that the products of the first four genes of the *cbl* operon, *cblA*, *cblB*, *cblC* and *cblD*, are sufficient for pilus assembly on the bacterial surface. Deletion of *cblB* abrogated pilus assembly and compromised the stability of the CblA protein in the periplasm. In contrast, deletion of *cblD* resulted in no pili, but there was no effect on expression and stability of the CblA protein subunit. These results, together with protein sequence homologies, predicted structural analyses, and the presence of typical amino acid motifs, are consistent with the assignment of functional roles for CblB as a chaperone that stabilizes the major pilin subunit in the periplasm, and CblD as the initiator of pilus biogenesis. It is also shown that expression of Cbl pili in *Escherichia coli* is not sufficient to mediate the binding of bacteria to the epithelial cell receptor cytokeratin 13, and that *B. cepacia* still binds to cytokeratin 13 in the absence of Cbl pili, suggesting that additional bacterial components are required for effective binding.

**INTRODUCTION**

Pili are surface appendages that allow bacteria to interact with each other and with the host. Pili are usually long rod-like fibres with a wide range of diameters (2–10 nm) and the pilus shaft is a polymer of the major pilin subunit, which is often used as a DNA marker to identify potentially virulent isolates. The biogenesis and surface assembly of functional pili is a complex process that involves the participation of several gene products. The biogenesis of the well-studied type I and P pili in uropathogenic *Escherichia coli* requires the function of 11 genes, whereas the formation and assembly of CFA/I and CS pili from enterotoxigenic *E. coli* requires only four genes. The genes that are usually involved in pilus biogenesis are those that encode a major pilin subunit, a periplasmic chaperone(s), a large outer-membrane protein, one or more minor pilin subunits and a regulatory protein(s) (Krogfelt, 1991).

*Burkholderia cepacia* is an opportunistic pathogen in cystic fibrosis (CF) patients and about 3–5% of CF patients are infected worldwide. At least nine different *Burkholderia* species form what is now known as the *B. cepacia* complex (Mahenthiralingam *et al.*, 2002). We have previously shown that *B. cepacia* isolate BC7, a member of an epidemic strain belonging to genovar IIIa (previously designated ET12), expresses long flexible type II pili with a novel cable morphology (Sajjan *et al.*, 1995). The gene that encodes the major pilin subunit of the Cbl pilus (*cblA*) has been cloned and sequenced, and its detection in CF isolates is presumed to be an indicator of virulence (Sun *et al.*, 1995). Although the *cblA* nucleotide sequence did not show homology to any of the known genes that encode major pilin subunits, the amino acid sequence of CblA was found to be 72–74% similar to the major pilin subunits of CFA/I and CS pili, respectively. Cbl pili differ from CFA/I and CS pili in receptor binding specificity, as binding is mediated by a 22 kDa protein associated with cable pili (Sajjan & Forstner, 1993; Sajjan *et al.*, 2000a). Usually, adhesins are minor protein subunits assembled on the tip of the pili and their genes are an integral part of the pilus gene cluster (Krogfelt, 1991; Sakellaris *et al.*, 1999). In some cases, the major pilin itself also functions as an adhesin (Bakker *et al.*, 1992; Irvin *et al.*, 1989; Jacobs *et al.*, 1987). To understand the

**Abbreviation:** CF, cystic fibrosis.

The GenBank accession number for the sequence determined in this work is AY082893.
mechanism of pili-mediated interactions of *B. cepacia* with respiratory cells, and to establish the role of cable pili in pathogenesis, it is essential to determine the organization and function of the *cbl* gene cluster. Ideally, this could be accomplished by the construction of *cbl* mutants in *B. cepacia*, but genomovar III isolates, in particular the clinical isolate BC7 expressing cable pili, are highly resistant to the majority of antibiotics used for genetic selection (Nzula et al., 2002). Therefore, we opted to reconstruct the assembly of cable pili in *E. coli* using recombinant plasmids carrying cloned genes of the *cbl* operon under the control of a regulated promoter. In this study, we demonstrate that four *cbl* genes from the cable pili gene cluster of *B. cepacia* BC7 are sufficient to direct the biogenesis and assembly of the pili in non-piliated *E. coli* cells. We also provide experimental evidence that the product of the *cblB* gene acts as a chaperone, while the product of the *cblD* gene is involved in the initiation of pilus biogenesis.

**METHODS**

**Bacterial strains and growth conditions.** *B. cepacia* isolates BC7 and J2315 (genomovar IIIa, ET12) were isolated from the sputum of CF patients and have been previously described (Sajjan et al., 1991, 2000a). *B. cepacia* isolates BC123 and BC124 are also genomovar IIIa clinical isolates kindly provided by Dr E. Tullis, St Michael’s Hospital, Toronto, Canada. *E. coli* DH5α and *E. coli* XL-1 Blue MR were purchased from Life Technologies and Stratagene respectively. For most experiments, bacteria were grown in LB broth (Difco). For pili expression and electron microscopy, bacteria were grown on LB agar at 37°C in the presence of appropriate antibiotics and inducer.

**Construction of a cosmid library and screening.** Genomic DNA from *B. cepacia* BC7 was partially digested with Sau3AI to give fragments of 30–45 kb. The fragments were ligated into SuperCosI vector DNA cleaved with *XbaI* and *BanHI*. The ligated DNA was packaged into lambda phage particles by using Gigapack III Gold kit (Stratagene) and transduced into *E. coli* XL-1 Blue MR. The cosmid library was screened by Southern blot hybridization with a digoxigenin-labelled *cblA* gene fragment, which was obtained by PCR using pUC18-52, as a DNA template (Sajjan et al., 1995).

**DNA manipulation methods and plasmids.** Synthetic oligonucleotides with an *XbaI* or *KpnI* site at the 5’ end were synthesized at the DNA synthesis facility, the Hospital for Sick Children, Toronto, Canada. DNA was amplified by PCR using the high-fidelity DNA polymerase *Pfu* Turbo (Stratagene) following the manufacturer’s instructions. Gene-specific primers synthesized with *KpnI* or *XbaI* were used to facilitate directional cloning into expression vectors. DNA ligations were carried out using the rapid DNA ligation kit from Roche Diagnostics. Plasmid DNA was transformed into *E. coli* by using CaCl2-treated cells as previously described (Brown et al., 1979). PCR amplification was used to detect the *cblA* gene in the clinical isolates BC123 and BC124 using chromosomal DNA as template and *cblA* gene-specific primers as described previously (Sajjan et al., 2000b).

The plasmids used in this study are listed in Table 1. Plasmids pMLBAD and pBAD18-Kan are low-copy-number expression vectors containing the *t*-arabinose-inducible BAD promoter, which have been described previously (Guzman et al., 1995; Lefebre & Valvano, 2002). Plasmid pUC18-52 contains a portion of the *cbl* operon and has been described previously (Sajjan et al., 1995). Plasmid pUS-2711 contains the first four genes of the *cbl* pili operon. This plasmid was created by digesting Cos-13, a cosmid clone containing the *cbl* operon, with *ApaI* (which cuts at *AlwII*) and *AolI* (Figs 1) into the pMLBAD vector, which was previously digested with *KpnI* and *XbaI*. This fragment was amplified using primers corresponding to the 5’ end of *cblA* (primer #17217; GATCGTACCATGCTGAAATGCGTTCCGA-9, *Tp*R, trimethoprim resistance; *Kan*R, kanamycin resistance; *Amp*R, ampicillin resistance.)

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<sup>a</sup>*Tp*R, trimethoprim resistance; Kan<sup>R</sup>, kanamycin resistance; Amp<sup>R</sup>, ampicillin resistance.
AGATCTAGATGCTTGTGGAAGATCTAAGAT, XbaI site underlined). The cblB gene was amplified using primers #15248 (GATCCGACCCATGGTCGTCGCTGAGCAATCGCGACGTCG; KpnI site underlined) and #17218 (GAGCTCAGAGTTAGTCCCTTCGTTGGTTGAAACAATGG; XbaI site underlined), while the cblD gene was amplified using primers #17219 (GATCTAAGCTTGCAGAGTCAGCTTGACAT, KpnI site underlined) and #15250 (also used for the construction of pUS-2802). Each of these fragments was cloned into pBAD18-Kan under control of the pBAD promoter to produce pUS-2828 (containing cblB) and pUS-2810 (containing cblD) (Fig. 1).

**Nucleotide sequencing and computer analysis.** Both strands of DNA from the various clones were sequenced using gene- and vector-specific primers by automated sequencing at the sequencing facility of the Hospital for Sick Children, Toronto. Manual sequencing to resolve regions with sequence ambiguities due to the high G+C content (68–70 mol%) in the B. cepacia genome was carried out with a T7 sequencing kit in conjunction with Deaza G/A dNTP mix (Amersham Pharmacia Biotech). Sequence data have been deposited in the GenBank under accession number AY082893.

**Cell fractionation.** E. coli or B. cepacia, isolate BC7, were grown on LB agar with or without 100 μg trimethoprim ml⁻¹ and 0-02 % l-arabinose overnight at 37˚C. Bacteria were harvested from the plate, suspended in 0-15 % sodium chloride and adjusted to 1 × 10¹⁰ c.f.u. ml⁻¹. Extracts containing pili sheared from the bacterial surface were prepared by incubation of the bacterial suspension (1 ml) at 60˚C for 20 min with shaking followed by centrifugation (Scott et al., 1992). Supernatant was collected and immediately mixed with complete protease inhibitor cocktail (Roche Diagnostics) followed by storage at −20˚C. Periplasmic proteins were extracted as previously described (Duthy et al., 2001). Briefly, bacteria (1 × 10¹⁰ c.f.u.) were suspended in 30 mM Tris/HCl pH 8-1 containing 20 % (w/v) sucrose and incubated with 0-1 M EDTA containing 100 μg lysozyme ml⁻¹ for 30 min on ice. Following centrifugation, supernatants were collected and stored at −20˚C. Whole-cell extracts were prepared by suspending bacteria (1 × 10¹⁰ c.f.u.) in 10 mM Tris/HCl, pH 6-8, containing 1 % SDS and 30 mM dithiothreitol (Bio-Rad), and boiling for 5 min, followed by centrifugation to clear cellular debris.

**Purification of cable pili.** Cable pili were isolated and purified from B. cepacia BC7 as described previously (Sajjan et al., 1995).

**Antibodies.** Production and characterization of anti-CblA serum has been previously described (Sajjan et al., 2002). CblD-specific antiserum was produced in rabbits by injecting a synthetic peptide, LLDKDGASREISD, which spans amino acids 292–306 of the predicted mature CblD protein. Antiserum was absorbed against E. coli extracts to remove non-specific antibodies.

**Western blot analysis.** Cell fractions (15 μl and 150 μl for detection of CblA and CblD respectively) were subjected to SDS-PAGE and proteins transferred onto Immobilon membranes. Blots were incubated with primary antibodies raised in rabbits and the bound antibody was detected by using anti-rabbit IgG conjugated with alkaline phosphatase and colour substrate NBT-BCIP.

**Binding assay.** Binding of bacteria to cytokeratin 13 was determined by bacterial overlay assay as described previously (Sajjan & Forstner, 1993; Sajjan et al., 2000a).

**Transmission electron microscopy.** Bacteria grown on agar plates were transferred to Formvar-coated grids and negatively stained with 1 % phosphotungstic acid as described previously (Sajjan et al., 2002; Sajjan & Forstner, 1993). Immunogold labelling of bacteria was carried out essentially as described earlier (Sajjan et al., 1995). Antibody specific to the major cable pilin subunit, CblA, was used at 1:50 dilution. The secondary antibody, conjugated with 10 nm gold particles, was used at 1:20 dilution. Grids were counterstained with 1 % phosphotungstic acid and observed under a JEOL 1200 EXII transmission electron microscope at 80 kV.

**Computer analysis.** Nucleotide sequences were analysed by using the BLAST Network Service at the National Center for Biotechnology Information. Putative signal peptides and their cleavage sites were determined on the SignalP V1.1 server at the Center for Biological Sequence Analysis (Nielsen et al., 1997). Computer-based
3D modelling of proteins was carried out on the 3D-PSSM server at the Biomolecular Modelling Laboratories, London, UK (Kelley et al., 2000).

RESULTS

Identification of the cbl gene cluster and predicted functions of cbl genes

Plasmid pUC18-52, was isolated from a genomic library constructed with random DNA fragments of B. cepacia BC7, which was screened by DNA–DNA hybridization using a probe fragment spanning a portion of the cbla gene (Sajjan et al., 1995). Sequencing of pUC18-52 revealed a complete gene downstream of cbla, and portions of two other genes (Fig. 1), suggesting that cbla is part of a larger gene cluster. To identify these other genes, we constructed a cosmid library from B. cepacia BC7 and screened it with a cbla-specific probe. Five colonies, out of 3–5 × 105 colonies screened, gave a positive hybridization signal. Restriction endonuclease fragments of cosmid DNA obtained from one of these colonies (clone 13) hybridized with probes generated from 3′ and 5′ sequences of pUC18-52 flanking the cbla gene. These fragments were subcloned and sequenced, spanning an 8054 bp region containing five ORFs, including the cbla gene (Fig. 1). The other ORFs were designated cblB, cblC, cblD and cblS (Fig. 1). All five genes were transcribed in the same direction and putative promoter sequences at -10 (ATCGAT) and -35 (CGAACCA) positions were identified upstream of cblB (from bp 216 to 245), suggesting an operon structure. The sequenced region has a G+C content of 64 mol% and carries no recognizable insertion sequences. The Sec system (Pugsley, 1993) appears to be responsible for the secretion of the Cbl proteins as they all possess typical signal sequences. Polypeptides encoded by cblB, cblC and cblD displayed 45–60 % similarity to analogous proteins of the CS1, CS2 and CFA/I pilus operons of enterotoxigenic E. coli (Sakellaris & Scott, 1998) and the TCF fimbrial operon of Salmonella typhi (Folkesson et al., 1999).

CblB, the first gene of the cbl operon, encodes a predicted mature protein of 26.3 kDa plus a 17 amino acid signal peptide. CblB has 30–34 % identity and 47–53 % similarity to the CooB, CotB and CFAB proteins of CS1, CS2 and CFA/I pili, respectively. Protein fold recognition analysis of CblB revealed an immunoglobulin-like fold similar to that of PapD in uropathogenic E. coli. CooB, CotB and PapD have been shown to function as periplasmic chaperones that interact with both major and minor pilin subunits and play a major role in pilus assembly (Hultegren et al., 1991; Sakellaris & Scott, 1998). The third ORF in the cable gene cluster, cblC, is located at the 3′ end of cbla. It consists of 2702 nucleotides spanning bases 1663 to 4365, and encodes a large polypeptide of 900 amino acids. The predicted mature CblC protein has a molecular mass of 93.4 kDa. A BlastP search combined with a search for conserved domains revealed an usher-like domain in the middle of the protein. Usher proteins are outer-membrane porin-like proteins and form a pore in the membrane for the transport of assembled pili (Thanassi et al., 1998). Thus, the features of CblC are consistent with the notion that this protein serves as an usher for cable pili assembly on the bacterial surface. The fourth gene in the cbl operon, cblD, spans bases 4441–5604 (Fig. 1) and encodes a polypeptide of 387 amino acids. The predicted molecular mass of mature CblD is 38.7 kDa after cleavage of a 27 amino acid signal peptide. The protein showed 31–33 % identity and 45–48 % similarity to analogous proteins of CFA/I (CFAE) and CS (CotD, CooD, CsoD) pili. These proteins have been shown to be minor pilin subunits required for initiation of pilus growth. The cblS gene encodes a predicted protein of 717 amino acids with a 34-residue signal peptide. This protein exhibited 40–46 % similarity to reported histidine kinase sensory proteins of two-component regulatory systems.

The biogenesis of cable pili in E. coli DH5α requires at least four cbl genes

In an attempt to identify the essential genes required for cable pili biogenesis, we cloned an Alw441–Alol fragment from cosmide clone 13, containing cblB, cbla, cblC and cblD, into pMLBAD (Lefebre & Valvano, 2002) to produce pUS-2711. This strategy placed the cblBACD genes under the control of the tightly regulated PBAD promoter from the araBAD (arabinose utilization) operon. E. coli DH5α transformed with pUS2711 was grown on LB agar containing 0.01 %, 0.02 % or 0.05 % arabinose and examined for the production of the major cable pilin subunit Cbla by screening bacterial colonies using a Cbla-specific antiserum (Sajjan et al., 2002). The optimal concentration of arabinose to induce the PBAD promoter without deleterious effects on colony morphology was found to be 0.02 % (data not shown). Hence, 0.02 % arabinose was used in all subsequent experiments.

The presence of Cbla was investigated in whole-cell extracts as well as in periplasmic and heat extracts, which were all subjected to Western blot analysis with Cbla-specific antiserum. Extracts prepared from B. cepacia BC7 and E. coli containing pMLBAD served as positive and negative controls, respectively. As expected, all three extracts from B. cepacia BC7 contained a strongly immunoreactive band at 15.8 kDa (Fig. 2). Extracts from E. coli DH5α (pMLBAD) did not show reactivity with anti-cbla serum. In contrast, the three fractions from E. coli DH5α (pUS-2711) displayed immunoreactive bands at 15.8 kDa similar to those found in the fractions from B. cepacia BC7. The band detected in periplasmic fractions from E. coli DH5α (pUS-2711) was considerably weaker than the bands from the other fractions. This was not due to overexpression of the Cbla protein in E. coli, since a similar weaker band was also detected in the periplasmic fraction of B. cepacia BC7. It is possible that these bands correspond to periplasmic Cbla subunits in transit to the cell surface. Alternatively, weak staining could be due to partial proteolytic degradation during the preparation of periplasmic protein extracts.
The surface location of pili with cable morphology in *E. coli* DH5α(pUS-2711) was further investigated by transmission electron microscopy. *E. coli* DH5α(pUS-2711) (Fig. 3c), but not *E. coli* DH5α(pMLBAD) (Fig. 3b), showed pili attached to the bacterial surface. The pili were long, flexible and intertwined with each other, similar to cable pili of *B. cepacia* BC7 (Fig. 3a). The small round vesicles observed in the background are drying artifacts and appear to occur with the use of Formvar film, since they were also present in control grids with bacteria that do not express pili. To confirm that the pili formed on *E. coli* DH5α(pUS-2711) were the product of the *cbl* genes, bacteria were subjected to immunogold labelling with CblA antiserum. Gold particles were observed all along the pilus fibres (Fig. 3d), indicating the presence of assembled CblA pilin subunits. *B. cepacia* BC7 showed similar reactivity with the CblA antiserum, whereas *E. coli* DH5α(pMLBAD) was negative (not shown). Taken together, the biochemical and morphological experiments described in this section demonstrate that the *cbl* genes carried on pUS-2711 are sufficient for the reconstruction of cable pili expression in *E. coli* DH5α.

### cblB and cblD are required for cable pili production

CblB has a predicted structure that suggests it plays a role as a periplasmic chaperone. We reasoned that if this is the case, the presence of CblB should be absolutely required for cable pili assembly. To test this hypothesis, we constructed pUS-2802 (Fig. 1), which lacks *cblB* but contains the *cblACD* genes. Extracts obtained from *E. coli* DH5α(pUS-2802) did not show immunoreactivity with anti-CblA serum, indicating the absence of CblA protein (Fig. 4a). Also, analysis by electron microscopy showed that *E. coli* DH5α(pUS-2802) cannot form pilus fibres (Fig. 5a). To confirm that the observed effect was due to the absence of *cblB*, pUS-2828, carrying a functional *cblB* under the control of the P_{BAD} promoter, was transformed into *E. coli* DH5α(pUS-2802). Fig. 4 shows that the presence of pUS-2828 restored the formation of CblA in all three fractions, as found with *E. coli* DH5α containing pUS-2711 (Fig. 2). The presence of CblA in a CblB-dependent manner also restored the production of pilus fibres on the bacterial surface as determined by electron microscopy (Fig. 5b). These results demonstrate that CblB is absolutely required for pilus formation.

To determine whether CblD is a minor protein associated with the cable pili, a purified pili preparation from *B. cepacia* BC7 was analysed by Western blot analysis using an anti-CblD antibody. A minor protein band was observed at 39 kDa, in agreement with the predicted molecular mass of the mature CblD protein (Fig. 6a). In addition, CblD was not detectable in *E. coli* DH5α containing the plasmid pUS-2802, which cannot express the putative CblB chaperone (Fig. 4b). These data indicate not only that CblD is a minor subunit of the pilus fibre, and present in low amounts relative to CblA, but also that expression of CblD requires the presence of a functional CblB protein.

To determine the role of CblD in pilus biogenesis, we utilized the *E. coli* system to reconstitute cable pili expression in the presence and absence of CblD. Thus, we constructed pUS-2831, which contains only the first three genes of the *cbl* operon (Fig. 1). Western blot analysis with anti-CblA serum revealed the presence of a 16 kDa polypeptide in whole-cell and periplasmic extracts, but not in the heat extract that should contain sheared pili (Fig. 6b). None of these fractions reacted with the CblD antiserum, indicating the absence of CblD in *E. coli* DH5α(pUS-2831) as expected. When *E. coli* DH5α(pUS-2831) was examined by transmission electron microscopy, no pili were observed on the cell surface (Fig 7a). Therefore, in the absence of CblD, CblA pilin is neither transported to the cell surface nor assembled into pilus fibres. The transformation of *E. coli* DH5α(pUS-2831) with pUS-2810, carrying a functional *cblD* gene under the control of the P_{BAD} promoter, resulted in the appearance of both CblA and CblD proteins in the heat extracts, as determined by Western blot analysis (Fig. 6c), indicating restoration of pilus assembly. This was confirmed by electron microscopy, which revealed pilus fibres on the bacterial surface (Fig. 7b). Thus, the results are consistent with the
notion that CblD is a minor pilin subunit that most likely participates in the initiation of pilus assembly.

Expression of cable pili is not sufficient to mediate binding to the cytokeratin 13 receptor

Previously we have demonstrated that *B. cepacia* isolates expressing cable pili bind to a 55 kDa epithelial cell receptor, cytokeratin 13, via a 22 kDa adhesin associated with the cable pili (Sajjan & Forstner, 1993; Sajjan et al., 2000a). Neither of the pilin subunits investigated in this study, CblA and CblD, corresponds to the molecular mass of the 22 kDa adhesin. Using a well-established overlay assay, we therefore tested whether cable-pili-expressing *E. coli* DH5α containing pUS-2711 were able to mediate binding to cytokeratin 13 (Sajjan & Forstner, 1993; Sajjan et al., 2000a). Fig. 8 shows a band of 55 kDa, corresponding to cytokeratin 13, in an overlay assay with *B. cepacia* BC7. In contrast, *E. coli*
DH5α(pUS-2711) did not bind to cytokeratin 13. These results suggest that cable pili formed by the functions of the cloned cblBACD genes are not sufficient to mediate binding to cytokeratin 13.

The failure of *E. coli* DH5α carrying the recombinant cblBACD genes to bind to cytokeratin 13 could imply that another component of the cable pili required for this function is absent in the *E. coli* host. Alternatively, the pilus proteins could be post-translationally modified to acquire cytokeratin 13 binding capacity in *B. cepacia* but not in *E. coli*. To distinguish between these two possibilities we investigated the cytokeratin 13 binding capacity of other *B. cepacia* clinical isolates currently in our collection that also belong to genomovar IIIa. Two isolates, *B. cepacia* BC123 and BC124, were found to carry cblA, but failed to express both major and minor pilin proteins CblA and CblD as determined by Western blot analysis using antisera to CblA and CblD respectively (Fig. 9a, b). Although isolates BC123 and BC124 did not show pili on their surface (data not shown), both of them exhibited binding to cytokeratin 13 by conventional overlay binding assay, although the binding was comparatively less than observed for isolate BC7 (Fig. 9c).

**Fig. 4.** Effect of the absence of the cblB gene on accumulation of CblA and CblD proteins in *E. coli* DH5α(pUS-2802) or *E. coli* DH5α(pUS-2802 + pUS-2828). Cell fractions were subjected to Western blot analysis using CblA (a) or CblD (b) antiserum. Molecular mass standards are in the left lane. WE, HE and PE represent whole-cell, heat and periplasmic extracts respectively.

**Fig. 5.** Transmission electron microscopy of (a) *E. coli* DH5α(pUS-2802) and (b) *E. coli* DH5α(pUS-2802 + pUS-2828). Bacteria were negatively stained as in Fig. 3. Bars, 500 nm.
DISCUSSION

In the present study, we have identified four new genes in the cbl operon. Comparisons with other pili systems, together with functional assays performed in this study, indicate that cblB, cblC and cblD encode proteins required for pili assembly, while cblS is predicted to encode the histidine kinase sensory component of a two-component regulatory system. The only potential promoter region was found upstream of cblB, suggesting that the cbl genes are organized as an operon. Therefore, we speculate that cblS may be involved in the regulation of cable pili gene expression. The gene arrangement of the first four genes of the cbl operon

Fig. 6. Detection of CblA and CblD in purified pili or E. coli extracts. Purified pili prepared from B. cepacia BC7 (1 or 20 μg), and extracts from either E. coli DH5×(pUS-2831) or E. coli DH5×(pUS2831 + pUS2810) were subjected to Western blot analysis using antisera to CblA or CblD (CblA ab or CblD ab). (a) Immunoreactivity of a purified pili preparation. The left lane represents molecular mass standards and the arrows on the right indicate the CblA (15-8 kDa) and CblD (39 kDa) bands. (b, c) Immunoreactivity of extracts from (b) E. coli DH5×(pUS2831) and (c) E. coli DH5×(pUS2831 + pUS2810) with antisera to CblD (top) and CblA (bottom). WE, HE and PE represent whole-cell, heat and periplasmic extracts respectively.

Fig. 7. CblD is required for surface expression of cable pili. Transmission electron micrographs of negatively stained E. coli DH5×(pUS-2831) (a) or E. coli DH5×(pUS2831 + pUS2810) (b). Bars, 500 nm.
in *B. cepacia* BC7 is identical to that found in the CS and CFA/I fimbrial operons present in enterotoxigenic *E. coli* (Sakellaris & Scott, 1998). Attempts to derive specific isogenic mutants of strain BC7 for identification of the function of individual *cbl* genes were unsuccessful due to the inherent resistance of isolate BC7 to a wide range of antibiotics. Hence, the cable operon was expressed in *E. coli* K-12 under the tight regulation of the PBAD promoter to study the function of two genes, *cblB* and *cblD*.

Although the organization of genes in the *cbl* operon and their predicted proteins are similar to the *E. coli* CS and CFA/I gene clusters and their products, the *cbl* operon differs in ways that may suggest an evolutionary difference. The CS1 and CFA/I gene clusters are encoded by plasmids, have a low G+C content compared with the *E. coli* genome and are flanked by insertion sequences, suggesting horizontal transfer of genes from another organism (Sakellaris & Scott, 1998). In contrast, the *cbl* operon is located in one or more chromosomes of *B. cepacia* (Lessie et al., 1996), has no recognizable flanking insertion sequences, and has a calculated G+C content similar to that of the *B. cepacia* genome, indicating that horizontal transfer of the *cbl* gene cluster is highly unlikely. These differences suggest independent acquisition of the pilus gene cluster in *E. coli* and *B. cepacia*, rather than a common ancestry.

We provide evidence that the biogenesis of the cable pilus requires only the expression of the first four genes of the *cbl* operon, *cblBACD*, similar to analogous CS and CFA/I pili.
of *E. coli* (Sakellaris & Scott, 1998). This conclusion is supported by the fact that *E. coli* K-12, which is non-piliated, was capable of expressing pili on its surface upon transformation with a plasmid encoding the four *cbl* genes. These pili were morphologically similar to cable pili expressed by *B. cepacia* isolate BC7 and reacted with Cba antisera (Sajjan et al., 1995). Based on the amino acid sequence similarities of CblB, CblC and CblD to analogous proteins of CS pili assembly, we predict that CblB may function as a periplasmic chaperone, CblC as an outer membrane usher, and CblD as a minor subunit involved in the initiation of pilus biogenesis. In the present study, we have provided functional evidence to confirm the role of CblB and CblD in pilus biogenesis.

A plasmid carrying only the *cblACD* genes failed to direct the production of pilus fibres. The CblA protein was not detected in any of the fractions examined. Theoretically, this could be due to instability of CblA in the absence of CblB, and partial proteolytic degradation in the periplasmic compartment by DegP and other proteases (Raivio & Silhavy, 2001). Following the reconstitution of the Cbl proteins by the addition of a functional *cblB* gene, the CblA protein reappeared in the periplasm concomitantly with the restoration of pilus fibres on the cell surface. A similar loss of CooA (the major pilin protein of CS1 pili) in the absence of CooB (a probable periplasmic chaperone) was observed by other investigators (Scott et al., 1992), and this effect was attributed to a polar effect in the *cooB* deletion mutant. Under our experimental conditions we do not expect such polar effects because the *cblACD* genes were all expressed under the control of the *pBAD* promoter located in the plasmid vector immediately upstream of *cblA*. CooB in CS1 pili was shown to interact with CooA in the periplasm, a process that was necessary for stabilization as well as for translocation of CooA to the outer-membrane protein CooC (Voegele et al., 1997). It is likely that similar interactions occur between CblB and CblA in the periplasm, and these are necessary to prevent proteolytic degradation of pilin subunits and to facilitate their translocation to the outer-membrane protein, CblC. In support of this notion, computer modelling revealed that CblB is structurally similar to a family of PapD-like pilus chaperones in having two predicted globular domains, which assume an overall topology of an immunoglobulin-like fold. The binding site in PapD comprises an arginine in position 8 and a lysine in position 112, and three alternating hydrophobic residues in a stretch beginning between the F1 and G1 β-strands, and extending into the G1 β-strand (Holmgren & Branden, 1989; Kuehn et al., 1993). The three alternating hydrophobic residues in PapD have been shown to interact directly with the β-zipper motif, GXYX₃HXHXH, present in the COOH terminus of the major and minor pilin subunits (Kuehn et al., 1993). Likewise, CblB has three conserved alternating hydrophobic residues in the same area, and both CblA and CblD subunits contain a β-zipper motif (GXYX₃HXHXH) in their COOH termini. Thus the parallel between PapD interaction with its pilin subunits and assumed CblB interactions with CblA and CblD is compelling.

CblD is an integral part of the pilus structure, because it was present in a purified pilus preparation obtained from *B. cepacia* BC7. *E. coli* K-12 cells containing a plasmid lacking *cblD* but encoding the *cblBAC* genes did not form pili. However, the CblA protein was detected in various cell fractions, demonstrating that the absence of pili was not due to the lack of stability of the CblA subunit. These observations suggest that CblD is not required for the stabilization of CblA, but may have another role in pilus biogenesis. Since it is found in the pilus fibre and is present in minute amounts compared to CblA, we propose that CblD functions in the initiation of pilus formation, as was shown in the case of its *E. coli* homologue CooD (Froehlich et al., 1994).

Minor pilin proteins CooD and CfaE, from CS1 and CFA/I pili, respectively, appear to mediate binding to intestinal epithelial cells and also to erythrocytes, causing haemagglutination (Sakellaris et al., 1999). Although *cblBACD* were sufficient to produce cable pili in *E. coli* K-12, the recombinant bacteria did not bind to the epithelial cell receptor cytokeratin 13 (Sajjan et al., 2000a), suggesting that the minor pilin subunit CblD is not the adhesin that mediates binding to cytokeratin 13. Alternatively, it is possible that *E. coli* K-12 may not process CblD properly and as a result, CblD no longer functions as an adhesin. This is an unlikely explanation, however, because some *B. cepacia* isolates of genomovar IIIa (such as strains J2315 and AU0007) express cable pili but do not bind to cytokeratin 13 (Sajjan et al., 2002). Furthermore, we show in this work that *B. cepacia* isolates BC123 and BC124, which also belong to genomovar IIIa, do not express CblA or CblD but still bind to cytokeratin 13. Thus, it is highly improbable that CblD mediates the binding of bacteria to cytokeratin 13. We cannot of course rule out the possibility that CblD may bind to another as yet uncharacterized receptor, but our conclusions are strengthened by the fact that the cytokeratin 13 binding adhesin has a molecular mass of 22 kDa (Sajjan & Forstner, 1993), whereas the CblD protein is 39 kDa. In contrast with previous assumptions, therefore, our present findings suggest that the 22 kDa adhesin protein may be encoded by a gene outside the *cbl* operon. The association of the adhesin with cable pili may occur only after both are expressed on the bacterial cell surface. Further studies are currently under way in our laboratories to identify the adhesin gene and to characterize the interaction of this protein with the cable pilus.

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