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The *dsbB* Gene Product Is Required for Protease Production by *Burkholderia cepacia*

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Burkholderia cepacia **KF1, isolated from a pneumonia patient, produces a 37-kDa extracellular metalloprotease. A protease-deficient and lipase-proficient mutant, KFT1007, was complemented by a clone having an open reading frame coding for a 170-amino-acid polypeptide which showed significant homology to *Escherichia coli* DsbB. KFT1007, a presumed *dsbB* mutant, also failed to show motility, and both protease secretion and motility were restored by the introduction of the cloned *dsbB* gene of *B. cepacia*. The mutant KFT1007 excreted a 43-kDa polypeptide that is immunologically related to the 37-kDa mature protease. These results suggested that the *dsbB* mutant secretes a premature and catalytically inactive form of protease and that disulfide formation is required for the production of extracellular protease by *B. cepacia*.**

Burkholderia cepacia is commonly found in the natural environment, such as in the soil, in rivers, and on plant surfaces. Once thought to be only a phytopathogen, the microorganism is now recognized as a major opportunistic pathogen of human disease, although relatively little concerning the pathogenesis of *B. cepacia* is known. The microorganism produces an extracellular metalloprotease which might be involved in pathogenesis, since intratracheal instillation of the protease causes bronchopneumonia in rats (13). Some other factors, such as lipase, hemolysin (1, 12), exopolysaccharide (20), iron-chelating siderophores (22), and cable-like appendage pilus fibers (21), are also produced by *B. cepacia.*

B. cepacia KF1 was isolated from a patient with severe pneumonia who was involved in a nosocomial outbreak (24, 27). This strain produces protease and causes experimental pneumonia in mice (26). To investigate the role of protease in *B. cepacia* pathogenesis, we have constructed a genetic analysis system for the microorganism and generated several protease-deficient mutants of strain KF1 (2). In the present study, one of the protease-deficient and lipase-proficient mutants was identified as having a transposon insertion in the homolog of *Escherichia coli dsbB*. The results of this study suggest that the disulfide formation is an essential process for production of the metalloprotease.

The bacteria and plasmids used in this study are listed in Table 1. A genomic plasmid library of *B. cepacia* KF1 was constructed with chromosomal DNA and a 13.9-kb *Escherichia-Burkholderia* shuttle vector, pTS1209 (2). *B. cepacia* KFT1007, a protease-deficient mutant of KF1, was transformed with the plasmid library by electroporation, and the resultant transformants were selected on protease indicator plates (16) supplemented with chloramphenicol (100 μg/ml). Plasmids isolated from chloramphenicol-resistant and protease-producing clones were introduced into *E. coli* DH10B, and a plasmid, designated pMA11, which conferred a stable protease-proficient phenotype to KFT1007 was obtained. By subcloning, the complementing region on pMA24 was reduced and was further reduced to the 0.7-kb region on pMA43 (Table 1).

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The quantitative determination of protease amounts was carried out according to the method described previously (19). Protease activities in culture supernatants of KFT1007 carrying recombinant plasmid revealed that pMA24 and pMA43 conferred to the mutant levels of activity comparable to or even higher than that of the wild-type KF1 (Table 2). Residual activities of KFT1007 and KFT1007 carrying the vector plasmid were undetectable on protease indicator plates.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (11) and immunoblotting with rabbit anti-protease antibody raised against *B. cepacia* KF1 protease were carried out (Fig. 1). The wild-type KF1 showed 43- and 34-kDa immunoreactive bands in addition to the 37-kDa mature protease band. In agreement with the protease activity (Table 2), the 37-kDa protease band was missing in the immunoblots of KFT1007 and KFT1007(pTS1209) but present in those of KFT1007(pMA24) and KFT1007(pMA43). On the other hand, the 43-kDa band was observed in most of the strains (Fig. 1).

Sequence analysis of the 1,303-bp *Mul*I fragment of pMA24 revealed an open reading frame extending from nucleotide position 583 to 1092 that had significant homology to that of *E. coli* disulfide bond oxidoreductase, DsbB (14), and the gene was designated *dsbB* of *B. cepacia*. The insertion site of the transposon in KFT1007 chromosomal DNA corresponded to the position 69 bases from the start codon of the *dsbB* gene.

The amino acid sequence of the putative *B. cepacia* DsbB was aligned with that of *E. coli* DsbB (14) and a putative *Pseudomonas aeruginosa* DsbB homolog (9) (Fig. 2). The overall homology of *B. cepacia* DsbB (170 amino acid residues) with *E. coli* DsbB (176 amino acid residues) is 24.7% identity and 55.3% similarity, and that with the putative *P. aeruginosa* DsbB (163 amino acid residues) is 28.8% identity and 68.8% similarity. Two pairs of cysteine residues which were identified as periplasmic essential residues for the activity of *E. coli* DsbB (7) are well conserved among the DsbB sequences; one is the Cys-41–X–X–Cys-44 configuration and the other is the Cys-102–$(X)_n$–Cys-130 configuration, where n = 27, 26, and 25 for *B. cepacia*, *P. aeruginosa*, and *E. coli*, respectively. No signal peptide-like sequence was found at the N terminus. Overall, DsbB of *B. cepacia* is a very hydrophobic protein. The protein has four possible membrane-spanning regions and arginine-rich sequences at the N and C termini. All these characteristics are similar to those of *E. coli* DsbB (14).

A mutant of *E. coli dsbB* fails to assemble functional flagella

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)[a]	Source or reference
B. cepacia		
KF1	Wild type	24
KFT1007	KF1 *dsbB*::Tn5-31Tp	2
E. coli		
DH10B	F$^-$ *araD139* Δ(*ara leu*)*7697* Δ*lacX74 galU galK rpsL deoR* ϕ80*dlacZ*ΔM15 *endA1 nupG recA1 mcrA* Δ(*mrr hsdRMS mcrBC*)	5
SK46	MC4100 derivative; F$^-$ *araD139* Δ(*argF-lac*)U169 *rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR phoR ppfA33*::Tn*5*[b]	S. Kamitani
SS143	KS272 derivative; F$^-$ Δ*lac-X74 galE galK rpsL* Δ*phoA dsbB*::*cam*[c]	S. Kishigami and K. Ito
Plasmids		
pTS1209	Cm^r Ap^r; *Escherichia-Burkholderia* shuttle vector	2
pMA11	Cm^r Ap^r; pTS1209 with 5-kb *Eco*RI fragment containing *dsbB*	This study
pMA24	Cm^r Ap^r; pTS1209 with 1.3-kb *Mlu*I fragment containing *dsbB′*[d]	This study
pMA43	Cm^r Ap^r; pTS1209 with 0.7-kb *Nru*I fragment containing *dsbB′*	This study
pMA48	Cm^r Ap^r; pTS1209 with 0.4-kb *Ava*II-*Mva*I fragment containing *dsbB′*	This study
pMA49	Cm^r Ap^r; pTS1209 with 0.4-kb *Mva*I-*Ava*II fragment containing *dsbB′*	This study

[a] Abbreviations for phenotypes: Ap^r, ampicillin resistance; Cm^r, chloramphenicol resistance.
[b] The *pffA33*::Tn*5* allele was introduced into MC4100 *phoR* by transduction from SK33 by the method of S. Kamitani et al. (8). The *pffA* gene is identical to *dsbA*.
[c] The *dsbB*::*cam* allele was introduced into KS272 (23) by transduction.
[d] A part of the C-terminal region of *dsbB* was deleted.

unless cystine is added, and this defect was ascribed to an inability to form disulfide bonds in the P-ring protein of flagellar basal bodies (4). Strain KFT1007 was found to be immotile in 0.3% agar containing a one-half concentration of dialyzed brain heart infusion medium (16), but motility was restored by the introduction of pMA24 or by the addition of 0.1 mM L-cystine to the agar. L-cystine was also effective at restoring protease production to *B. cepacia* KFT1007. The immotility phenotype of a *dsbB* mutant, *E. coli* SS143, but not that of a *dsbA* mutant, strain SK46, was complemented by introducing *B. cepacia dsbB* (data not shown), indicating that *B. cepacia* DsbB is active in the heterologous host.

The DsbA-DsbB system appears to be widespread in gram-negative bacteria. Homologs of DsbA have been found in *Vibrio cholerae* (17) and *Haemophilus influenzae* (25). The absence of DsbA in *V. cholerae* results in the absence of secretion of the cholera toxin and of a major protease (17). The secretion of pullulanase by *Klebsiella oxytoca* proceeds via a two-step SecA-dependent mechanism, and DsbA is required for its correct folding in the periplasm and might be necessary for its secretion through the outer membrane (18).

TABLE 2. Protease activity in culture supernatants of various *B. cepacia* strains[a]

Strain	Protease activity[b] (U/ml)	Protein (μg/ml)	Sp act (U/μg)
KF1	220	38	5.8
KFT1007	12	31	0.4
KFT1007(pTS1209)	15	52	0.3
KFT1007(pMA24)	255	46	5.5
KFT1007(pMA43)	426	57	7.4
KFT1007(pMA48)	65	58	1.1
KFT1007(pMA49)	7	58	0.1

[a] Cells were grown in dialyzed brain heart infusion medium (16).
[b] Aliquots (3 ml) of reaction mixture containing 10 mg of hide powder azure (Sigma), 50 mM sodium acetate buffer (pH 6.0), 1 mM $CaCl_2$, and appropriate amounts of culture supernatant were incubated at 37°C for 1 h. After incubation, the A_{595} of the supernatant from the reaction mixture was determined. One unit was defined as the amount of enzyme that, after a 1-h incubation at 37°C, produced a change of 0.25 in the optical density at 595 nm.

In SDS-PAGE immunoblot experiments, the culture supernatant of the wild-type *B. cepacia* strain as well as its isogenic *dsbB* mutant KFT1007 supplemented with *dsbB*-carrying plasmid showed a discrete 43-kDa band in addition to the mature 37-kDa protease band. Since the former was observed also with *dsbB*-defective strains, it is tempting to speculate that the 43-kDa precursor protein secreted into the periplasm is converted to the active 37-kDa protease which is assisted by the DsbA-DsbB system. *B. cepacia* 715j produces two immunologically related proteases; the major, 36-kDa protease is highly active, whereas the minor, 40-kDa protease has a very low level of activity (10). We carried out immunoblotting of the culture supernatant of strain 715j with anti-KF1 protease, which was indistinguishable from that of strain KF1 (data not shown). These results suggested that the 40-kDa protease of Kooi et al. (10) is similar, if not identical, to the 43-kDa protein.

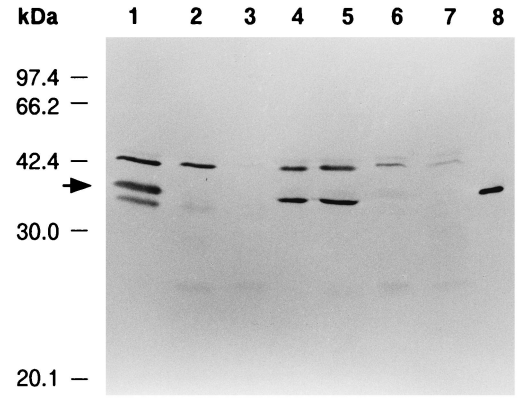

FIG. 1. Immunoblot analysis, using anti-KF1 protease, of culture supernatants of *B. cepacia* KF1, KFT1007, and KFT1007 carrying various plasmids. The molecular mass standards were phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), aldolase (42.4 kDa), carbonic anhydrase (30.0 kDa), and trypsin inhibitor (20.1 kDa). The position of the purified protease (37 kDa) is shown by an arrow. Lane 1, KF1; lane 2, KFT1007; lane 3, KFT1007(pTS1209); lane 4, KFT1007(pMA24); lane 5, KFT1007(pMA43); lane 6, KFT1007(pMA48); lane 7, KFT1007(pMA49); lane 8, purified KF1 protease (80 ng).

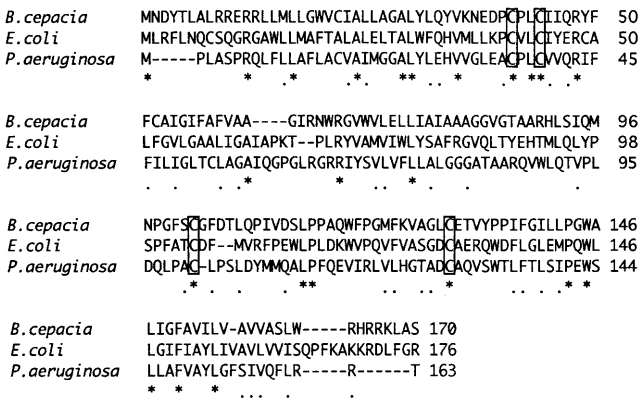

FIG. 2. Sequence alignment of the deduced amino acid sequence of DsbB of *B. cepacia* with that of *E. coli* and the putative DsbB of *P. aeruginosa*. Residues identical in all sequences and conserved residues are indicated by asterisks and dots, respectively. The four conserved cysteine residues are boxed.

B. cepacia DSM3959 lipase is secreted through the inner membrane after cleavage of a signal peptide (6). In the periplasm, prelipase is folded to an active state by a molecular chaperon, LimA, encoded by *limA* (6). The disulfide bond formation system appears not to be required for this process, since *dsbB*-defective KFT1007 showed a lipase-proficient phenotype. Although a signal peptide in *B. cepacia* protease has not been identified, it is plausible to assume that preprotease is secreted into the periplasm, folded by the help of the disulfide bond formation system, processed, and excreted through the outer membrane. This assumption was supported by our recent observations that several protease-deficient mutants of *B. cepacia* KF1 isolated by transposon mutagenesis were also lipase deficient (2) and that these mutants had the transposon in the chromosomal region encoding homologs for the general secretion pathway of *P. aeruginosa* (3, 15; also unpublished data).

Nucleotide sequence accession number. The nucleotide sequence of *dsbB* from *B. cepacia* KF1 has been deposited in the DDBJ, EMBL, and GenBank databases and has been given the accession number D83234.

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