An extracellular zinc metalloprotease gene of *Burkholderia cepacia*


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*Burkholderia cepacia* produces at least one extracellular zinc metalloprotease that may be involved in virulence. A *B. cepacia* zinc metalloprotease was cloned using a *Burkholderia pseudomallei* zinc metalloprotease gene as a probe. The predicted amino acid sequences of these *B. cepacia* and a *B. pseudomallei* extracellular zinc metalloproteases indicate that they are similar to the thermolysin-like family of metalloproteases (M4 family of metalloendopeptidases) and are likely to be secreted via the general secretory pathway. *zmpA* isogenic mutants were constructed in *B. cepacia* genomovar III strains Pc715j and K56-2 by insertional inactivation of the *zmpA* genes. The *zmpA* mutants produced less protease than the parent strains. The *B. cepacia* strain K56-2 *zmpA* mutant was significantly less virulent than its parent strain in a chronic respiratory infection model; however, there was no difference between the virulence of *B. cepacia* strain Pc715j and a Pc715j *zmpA* mutant. The results indicate that this extracellular zinc metalloprotease may play a greater role in virulence in some strains of *B. cepacia*.

INTRODUCTION

*Burkholderia cepacia* and *Burkholderia pseudomallei* are Gram-negative opportunistic pathogens belonging to the β-subclass of the phylum *Proteobacteria* (Yabuuchi, 1992). *B. pseudomallei* is a soil saprophyte and the causative agent of melioidosis, a glanders-like disease of humans and animals (Dance, 1991). Infection is most likely due to soil or water contamination of skin abrasions or inhalation of contaminated sources. Individuals more susceptible to *B. pseudomallei* infection include diabetics, alcoholics and those with chronic renal failure (Chaowagul et al., 1989).

*B. cepacia* is naturally found in soil and water. Strains originally identified as *B. cepacia* have been classified into at least nine genomovars, which are referred to as the *B. cepacia* complex (Vandamme et al., 1997; Vermis et al., 2002). Colonization of cystic fibrosis (CF) patients with *B. cepacia* complex organisms can lead to chronic airway infection and increase morbidity and mortality in these patients. These infections occasionally result in a rapid pulmonary decline associated with septicaemia, which may result in death, often referred to as ‘cepacia syndrome’ (reviewed by Mohr et al., 2001).

Sixty-nine to 88% of clinical *B. cepacia* isolates produce proteases (Gessner & Mortensen, 1990; Gilligan, 1991; McKevitt & Woods, 1984; Nakazawa et al., 1987). A recent study by Gotschlich et al. (2001) reported that strains of *B. cepacia* genomovars I and III, and *Burkholderia stabilis* are positive for extracellular protease activity, whereas strains of *B. cepacia* genomovar VI, *Burkholderia multivorans* and *Burkholderia vietnamiensis* do not have detectable extracellular protease activity (Gotschlich et al., 2001). In Canada, approximately 80 % of CF isolates are classified as genomovar III and 9-3 % are classified as *B. multivorans* (Speert et al., 2002). In the United States 50 % of CF isolates are classified as genomovar III organisms, 38 % are classified as *B. multivorans* and 5 % are classified as *B. vietnamiensis* (LiPuma et al., 2001).

A 36 kDa zinc metalloprotease, originally designated PSCP (*Pseudomonas cepacia* protease), has been described in *B. cepacia* genomovar III strain Pc715j (McKevitt et al., 1989). McKevitt et al. (1989) demonstrated that this zinc metalloprotease is capable of cleaving gelatin, hide powder and human collagen types I, IV and V (McKevitt et al., 1989). Biochemical evidence indicates that PSCP is a zinc metalloprotease since it is inhibited by 0-1 mM EDTA and 0-1 mM 1,10-phenanthroline. This inhibition is reversible by the addition of zink and calcium salts (McKevitt et al., 1989). PSCP may play a role in the virulence of *B. cepacia*. McKevitt et al. (1989) demonstrated that purified PSCP induces bronchopneumonia in rat lungs characterized by polymorphonuclear leukocyte infiltration and proteinaceous exudate in the airways. Immunization with a peptide corresponding to a conserved zinc metalloprotease epitope...
significantly decreased the severity of experimental *B. cepacia* lung infections (Sokol *et al.*, 2000). Further study of the role of extracellular proteases in the pathogenesis of *B. cepacia* infections has been limited by the lack of isogenic protease mutants.

*Bacillus thermoproteolyticus* thermolysin was the first zinc metalloprotease for which the three-dimensional structure was determined (Colman *et al.*, 1972). Consequently, thermolysin has become a model for the zinc metalloproteases belonging to the M4 peptidase family, also known as the thermolysin-like metalloproteases (Rawlings & Barrett, 1995). In this study the thermolysin sequence was used to search for a homologue in the *B. pseudomallei* K96243 genome sequence (www.sanger.ac.uk/projects/B_pseudomallei/blast_server.shtml) in an effort to identify a zinc metalloprotease gene in *B. pseudomallei*. The *B. pseudomallei* zinc metalloprotease gene was then used to identify a homologue in *B. cepacia*. The role of this *B. cepacia* zinc metalloprotease in virulence was investigated.

### METHODS

#### Bacterial strains, plasmids and culture conditions. 

Strains and plasmids used in this study are listed in Table 1. For genetic manipulations, bacterial cultures were grown at 37 °C in Luria–Bertani (LB) (Life Technologies) or Bacto-terrific broth (Difco). For the virulence studies cultures were grown overnight at 32 °C in dialysed-tryptic soy broth (Difco) treated with Chelex-100 (Bio-Rad) (Ohman *et al.*, 1980). Trypticase soy agar (TSA) (Difco) and *Bacillus pseudomallei* selection agar (Henry *et al.*, 1997) were used to quantify bacteria in lung homogenates. For protease production, cultures were grown in 0-25 % trypticase soy broth (Difco) with 5 % Bacto-peptone (PTSB). For growth curves, cultures were grown in PTSB or M9 (Sambrook, 1989) supplemented with 0-1 % casein. When required, antibiotics were used at the following concentrations: for *B. cepacia* tetacycline (Tc) 250 μg ml⁻¹, trimethoprim (Tp) 100 μg ml⁻¹; and for *Escherichia coli*, 100 μg ampicillin ml⁻¹ (Ap), 15 μg Tc ml⁻¹ and 1·5 mg Tp ml⁻¹.

#### DNA manipulations. 

Molecular biology techniques were generally performed as described by Sambrook *et al.* (1989). Genomic DNA was isolated from *B. cepacia* as described by Ausubel *et al.* (1989) and from *B. pseudomallei* using the Wizard Genomic DNA

### Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference(s) or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
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<tr>
<td><em>E. coli</em></td>
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</tr>
<tr>
<td>DH10b</td>
<td>F⁻ mcrA Δ(mrr-hsdRMS-mcrBC) ΔlacZM15 ΔlacX74 deoR recA1 endA1 araA139 Δ(ara, leu)7697 gaiU galKΔ7 rpsLΔ1 mutG</td>
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<tr>
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<td>F⁻ ΔlacZM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hsdR17(rK2m),m1 ΔpaoA ΔsupE44'' thi-1 gyrA96 relA1</td>
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<tr>
<td>TOP10</td>
<td>F⁻ mcrΔ(ΔlacZM15Δ(lacZYA-argF)U169ΔdeoRΔrecA1ΔendA1ΔhsdR17(rK2m),m1ΔpaoAΔsupE44''Δthi-1ΔgyrA96ΔrelA1)Δ(ara,leu)7697ΔgaiUΔgalKΔ7ΔrpsLΔ1ΔmutG</td>
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<tr>
<td><strong>B. pseudomallei</strong></td>
<td></td>
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</tr>
<tr>
<td>1026b</td>
<td>Clinical isolate (human); SmR TcS</td>
<td>DeShazer &amp; Woods (1996)</td>
</tr>
<tr>
<td>K56-2</td>
<td>CF sputum isolate; genovar III</td>
<td>Darling <em>et al.</em> (1998); Mahenthiralingam <em>et al.</em> (2000)</td>
</tr>
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<td>K56-2-9</td>
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<td>This study</td>
</tr>
<tr>
<td>Pc715j-72</td>
<td>Pc715j derivative; zmpA::Tp</td>
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<td>pRK2013</td>
<td>ColE1 Tra (RK2) KmR</td>
<td>Figurski &amp; Helinski (1979)</td>
</tr>
<tr>
<td>p34E-Tp</td>
<td>Source of <em>tp</em> cassette; TpR</td>
<td>DeShazer &amp; Woods (1996)</td>
</tr>
<tr>
<td>p34S-Tc</td>
<td>Source of <em>tc</em> cassette; TcR</td>
<td>Dennis &amp; Zylstra (1998)</td>
</tr>
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<td>pEX18Tc</td>
<td>Suicide vector; sacB TcR</td>
<td>Hoang <em>et al.</em> (1998)</td>
</tr>
<tr>
<td>pCC12</td>
<td>pEX18Tc with 2·6 kb <em>PstI</em> fragment from Pc715j containing zmpA gene; TcR</td>
<td>This study</td>
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<tr>
<td>pCC12T</td>
<td>pCC12 with zmpA inactivated with <em>tp</em> cassette; TcR TpR</td>
<td>This study</td>
</tr>
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<td>pUCP26</td>
<td>Broad-host-range vector; TcR</td>
<td>West <em>et al.</em> (1994)</td>
</tr>
<tr>
<td>pCC13</td>
<td>pUCP26 with 2·6 kb <em>PstI</em> fragment from Pc715j containing zmpA gene; TcR</td>
<td>This study</td>
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<tr>
<td>pUCP28T</td>
<td>Broad-host-range vector containing <em>oriT</em> for conjugal transfer; TpR</td>
<td>Schweizer (1996)</td>
</tr>
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<td>pUCP28PTc</td>
<td>pUCP28T with a TcR cassette inserted in the HindIII restriction site of the multiple cloning site; TpR TcR</td>
<td>This study</td>
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<tr>
<td>pCC14</td>
<td>pUCP28Tc with 2·6 kb <em>PstI</em> fragment from Pc715j containing zmpA gene; TpR TcR</td>
<td>This study</td>
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</tbody>
</table>
Cloning zmpA from B. pseudomallei. The B. thymoproteolyticus thermolysin sequence (accession no. X76986) was used to identify a thermolysin-like zinc metallopeptase gene (zmpA) homologue in the B. pseudomallei K96243 genome sequence at the Wellcome Trust Sanger Institute (www.sanger.ac.uk/projects/B_pseudomallei_blast_server.shtml) (Nielsen et al., 1997) was employed. Custom oligonucleotides were synthesized by Invitrogen Life Technologies. The zmpA gene was identified by colony hybridization (Woods, 1984) and designated pCC12.

DNA sequencing and sequence analysis. The nucleotide sequences of the zmpA genes were determined using the T7 and M13R universal primers and primers designed to the partially determined sequence. Custom oligonucleotides were synthesized by Invitrogen Life Technologies. Nucleotide sequencing was conducted using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit with Ampli-Taq DNA polymerase (Perkin-Elmer) and analysed with an ABI373A DNA sequence by University Core DNA Services (University of Calgary). Sequences were analysed using the gapped BLASTX and BLASTP programs (Altschul et al., 1997) and DNAMAN software (Lynnon Biosoftware). SignalP V1.1 (www.cbs.dtu.dk/services/SignalP/#submission) (Nielsen et al., 1997) was employed for the identification of putative signal sequence cleavage sites. Alignments were conducted using the CLUSTALX program (www.ebi.ac.uk/clustalw/index.html).

N-terminal sequencing of PSCP. B. cepacia PSCP was purified as described previously (Kooi et al., 1994; McKevitt & Woods, 1984) and partial N-terminal amino acid microsequencing of PVDF-electroblotted PSCP was performed using an ABI sequencer at the Department of Biochemistry, University of Victoria (UVic-Genome BC Proteomics Center).

zmpA mutant construction. A trimethoprim cassette was inserted into the BstWI restriction site of B. cepacia zmpA, resulting in plasmid pCC12T. Triparental matings were performed using E. coli DH5a (pRK2013) (Figurski & Helinski, 1979) to mobilize pCC12T from E. coli DH10b into B. cepacia Pc715 or K56-2. The insertional inactivation of zmpA was confirmed by PCR or by Southern hybridization analysis. Mutants were confirmed to have the same enzymic features were observed for the deduced amino acid sequence of the B. pseudomallei K96243 genome sequence using the TBLASTN search tool (Madden et al., 1996). The gene was cloned from B. pseudomallei 1026b via PCR and used as a probe to identify the gene encoding an extracellular metallopeptase of B. cepacia. The product of the B. cepacia zmpA ORF was predicted to be 565 aa in length and to have a molecular mass of 59 806 Da. The deduced amino acid sequence revealed a putative signal peptide cleavage site at aa 24 and a 13 aa region, 218-AAATGGRSLYY-230, identical to the partial N-terminal amino acid sequence determined for mature PSCP. Processing at this site would yield a peptide of 36 857 Da with a predicted pl of 6.3 (http://ca.expasy.org/cgi-bin/protparam). This is consistent with the molecular mass of B. cepacia PSCP (36 000 Da) observed by SDS-PAGE analysis (Kooi et al., 1994). Similar features were observed for the deduced amino acid sequence.
of *B. pseudomallei* ZmpA as illustrated in Fig. 1. The above properties indicate that the proteases have a preproenzyme structure similar to other zinc metalloproteases such as *Pseudomonas aeruginosa* elastase, *Bacillus thermoproteolyticus* thermolysin and *Vibrio cholerae* HA/protease (Bever & Iglewski, 1988; Hase & Finkelstein, 1991; O'Donohue & Beaumont, 1996).

Analysis of the deduced amino acid sequences of the *B. cepacia* and *B. pseudomallei* proteases reveals two conserved sequence characteristics of the thermolysin-like family of metalloproteases (M4 family of metallopeptidases) (Rawlings & Barrett, 1995), the zinc metalloprotease active-site motif (HExxH) at aa residues 376-HEMSH-380 and 396-GGLNESTSD-404, which contains the third zinc ligand (aa residue 400-E). The presence of these conserved residues suggests that these proteases belong to the thermolysin-like metalloproteases. Interestingly, biochemical evidence demonstrates that PSCP is inhibited by the metalloprotease inhibitors EDTA and 1,10-phenanthroline (McKevitt et al., 1989), but it is not inhibited by phosphaomidon, an inhibitor of thermolysin (data not shown).

Recently, the genomic sequence of *B. cepacia* J2315 has been completed by the Wellcome Trust Sanger Institute (www.sanger.ac.uk/Projects/B_cepacia/). The J2315 *zmpA* gene is 99-95 % identical to Pc715j *zmpA*. There is a single amino acid change at residue 489 from threonine to serine in Pc715j. Although the K56-2 *zmpA* gene was not sequenced it belongs to the same ET12 clone of genomovar III as J2315 (Mahenthiralingam et al., 2000). A putative transcriptional start site was identified upstream of *zmpA* in the J2315 and the Pc715j nucleotide sequence (Neural Network Promoter Prediction; www.fruitfly.org/seq_tools/promoter.html). This suggests that the transcription of *zmpA* is under the control of a promoter immediately upstream of the gene.

**Effects of zmpA mutations on protease expression**

Mutations in the *zmpA* genes were constructed by insertion inactivation of *zmpA* with a trimethoprim cassette, followed by allelic exchange into strains Pc715j or K56-2, resulting in Pc715j-*zmpA* and K56-2-*zmpA*. The predicted signal peptidase cleavage site is underlined with a dotted line and the mature N-terminal amino acid sequence of PSCP is boxed (solid line). The conserved zinc metalloprotease motif, HExxH, is boxed with a dotted line and a second consensus sequence, GGLNESTSD, is underlined (solid line). Bold letters represent strictly conserved residues of the thermolysin-like metalloproteases. '*' indicates identical residues; ':' indicates conserved residues; '.' indicates semi-conserved residues.
and Western Immunoblots a 36 kDa protein was present in the zmpA mutant that reacted with antibodies to PSCP (Fig. 2b). Similar results were observed in Pc715j (data not shown). Interestingly, the insertion of the Tp cassette at base 1066 results in translation of a protein with a predicted molecular mass of 36 268 Da. This consists of the propptide and the truncated mature protein to the stop codon within the trimethoprim cassette. This fusion protein has a predicted pI (6.8) that is higher than that of the mature PSCP enzyme (6.3). (http://ca.expasy.org/cgi-bin/pi_tool). Therefore, two-dimensional gel electrophoresis was used to examine the extracellular protein profiles of Pc715j and Pc715j-72 (Fig. 3a, b). In the Western immunoblot of Pc715j extracellular proteins a 36 kDa protein corresponding to PSCP was detected by mAb 36-6-6 (Fig. 3c) whereas a 36 kDa protein with a higher isoelectric point than PSCP was detected with mAb 36-6-6 in the immunoblot of Pc715j-72 (Fig. 3d). We hypothesize that this peptide is the unprocessed propptide with the truncated mature peptide that has a predicted pI of 6.8 compared to the predicted pI of 6.3 for the mature PSCP. The mAb 36-6-6 reacts with epitopes surrounding the active site of P. aeruginosa elastase (Kooi et al., 1997). Although the strongest reacting epitope (peptide 15) of elastase is absent in this truncated fusion protein, some of the other reactive epitopes are present. This explains why we observed an extracellular protein in the zmpA mutant strains of B. cepacia that migrated at 36 kDa on one-dimensional SDS-PAGE and reacted with mAb 36-6-6. This fusion protein has no proteolytic activity since the active site has been interrupted. A 40 kDa protein was also detected in Western immunoblots of Pc715j, Pc715j-72 (Fig. 3), K56-2 and K56-2-9 (Fig. 2) as previously observed by Kooi et al. (1994). In Pc715j a 20 kDa protein was also detected on the immunoblots. Although this corresponds in size to the 20 kDa protein present in supernatants of K56-2, the K56-2 protein did not react with mAb 36-6-6 and was predicted to be the pro-region of PSCP (Fig. 2). It is not clear if the 20 kDa protein in Pc715j is a degradation product of PSCP or an immunologically related protein.

The proteolytic activities of the mutants were compared to their parent strains using skim milk agar and hide blue azure as substrates. On skim milk agar Pc715j(pUCP28Tc) produced 4.3±0.3 mm zones of clearing compared to 3.3±0.3 mm in the zmpA mutant, Pc715j-72(pUCP28Tc) and 5.0±0.0 mm in Pc715j-72(pCC14). The difference in zone size between the three strains was significant (P<0.05; ANOVA). There was a more dramatic difference in the zone sizes of K56-2 and its zmpA mutant, K56-2-9, which produced little or no zones of clearing on skim milk agar after 24 h. K56-2(pUCP26) produced 3.2±0.6 mm zones of clearing compared to 0.5±0.0 mm by K56-2-9 (pUCP26) and 2.7±0.3 mm by K56-2-9(pCC13). When

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**Fig. 2.** Extracellular protein profiles of K56-2(pUCP26), K56-2-9(pUCP26) and K56-2-9(pCC13). (a) SDS-PAGE (12.5%) of TCA-precipitated cell-free supernatants. Lanes: 1, pre-stained molecular mass markers; 2, K56-2(pUCP26); 3, K56-2-9 (pUCP26); 4, K56-2-9(pCC13); 5, PSCP; 6, 40 kDa protein. (b) Western immunoblot of TCA-precipitated cell-free supernatants. Lanes: 1, pre-stained molecular mass markers; 2, K56-2(pUCP26); 3, K56-2-9(pUCP26); 4, K56-2-9 (pCC13); 5, PSCP.
hide blue azure was used as a substrate, the cell-free supernatant of Pc715j-72(pUCP28Tc) contained 53% less proteolytic activity than Pc715j(pUCP28Tc), whereas Pc715j-72(pCC14) demonstrated a 3.6-fold increase in the amount of proteolytic activity compared to the wild-type Pc715j(pUCP28Tc) (data not shown). As previously reported K56-2 cell-free supernatants did not contain sufficient proteolytic activity to be quantified in this assay (Lewenza et al., 1999).

The growth of the zmpA mutants was compared to their respective wild-type strains in a rich medium, PTSB, and in M9 minimal medium supplemented with 0.1% casein (data not shown). There were no growth rate differences between the B. cepacia zmpA mutants and their wild-type strains in either medium, indicating that the decrease in proteolytic activity observed in the zmpA mutants is not due to decreased growth. API20 E systems were used to examine the enzymic activities of the parent and mutant strains. The profile of the Pc715j zmpA mutant was identical to Pc715j, with the exception that it did not cleave gelatin. Neither K56-2 nor K56-2-9 cleaved gelatin in this system.

Relative virulence of the B. cepacia zmpA mutants

Rats were infected with B. cepacia Pc715j, K56-2 and their respective mutants, and on days 7 and 14 p.i. quantitative histopathologic and bacteriologic analyses were performed on the lungs. On days 7 and 14 p.i. the number of bacteria (c.f.u. ml⁻¹) recovered from the lungs of rats infected with K56-2-9 was approximately 4 logs lower than the parent strain (Table 2). Interestingly, the K56-2-9 zmpA mutant was only recovered from one of three animals on day 7 p.i. and two of four animals on day 14 p.i. These results indicate that the B. cepacia K56-2-9 zmpA mutant is less able to persist in the lung than the B. cepacia K56-2 parent strain, which had similar numbers of bacteria recovered on both days 7 and 14 p.i. There was no difference between the numbers of B. cepacia Pc715j and Pc715j-72 recovered from the lungs of rats on day 7 or 14 p.i. (Table 2). This indicates that both Pc715j and Pc715j-72 were able to persist within the lung and the production of PSCP was not necessary for persistence in this strain.

Rats infected with K56-2-9 had significantly less lung...
pathological changes than rats infected with K56-2 on both days 7 and 14 p.i. (P<0.01; Student’s unpaired t test). In contrast, there were no significant differences in the degree of lung pathology observed between \( B. \text{cepacia} \) Pc715j and Pc715j-72 on either day 7 or day 14 (Table 2). These data suggest that PSCP may be an important virulence factor in some strains of \( B. \text{cepacia} \), but production of additional proteases may compensate for a loss of PSCP in strains such as Pc715j.

**DISCUSSION**

In this study we identified genes from \( B. \text{cepacia} \) and \( B. \text{pseudomallei} \) that encode zinc metalloproteases. The deduced amino acid sequences of the \( B. \text{cepacia} \) and \( B. \text{pseudomallei} \) extracellular zinc metalloproteases have a preproenzyme structure similar to members of the thermolysin-like metalloprotease family (Hase & Finkelstein, 1991; Kessler & Safrin, 1988; McIver et al., 1991). The preproenzyme structure dictates secretion through the general secretory pathway (Pugsley, 1993). The signal peptide directs secretion through the periplasmic membrane, followed by cleavage of the signal peptide on the outer side of the periplasmic membrane (Pugsley, 1993). The pro-sequences of \( P. \text{aeruginosa} \) elastase (LasB) and \( Bacillus \) thermoproteolyticus thermolysin act as molecular chaperones that mediate folding of the mature enzymes within the periplasm (Braun et al., 1996; Marie-Claire et al., 1999; McIver et al., 1995; O’Donohue & Beaumont, 1996). The propeptides remain non-covalently associated with the mature enzymes to inhibit their proteolytic activity until liberation from the cell (Braun et al., 1998; Kessler & Safrin, 1994; Kessler et al., 1998; O’Donohue & Beaumont, 1996).

The prediction that \( B. \text{cepacia} \) PSCP is secreted via the general secretory system agrees with the findings of Nakazawa & Abe (1996). Furthermore, they determined that protease production was dependent on DsbB, suggesting that \( B. \text{cepacia} \) protease is secreted via the general secretory pathway and involves disulfide bond formation (Abe & Nakazawa, 1996; Nakazawa & Abe, 1996). Anti-PSCP mAbs react with PSCP and a 40 kDa protein present in \( B. \text{cepacia} \) Pc715j cell-free supernatants (Kooi et al., 1994). The 40 kDa protein preparation demonstrated weak proteolytic activity and could potentially be a precursor of PSCP or an immunologically related protein (Kooi et al., 1994). Abe & Nakazawa (1996) predicted that a 43 kDa protein (likely to be the 40 kDa described by Kooi et al., 1994) was perhaps a precursor to a 37 kDa protease secreted by \( B. \text{cepacia} \) KF1. In this study we determined that the precursor to PSCP has a predicted molecular mass of approximately 59-8 kDa and therefore it is unlikely that the 40 kDa protein is a precursor to PSCP. Also, the extracellular protein profiles of the \( B. \text{cepacia} \) zmpA mutant strains still contain the 40 kDa protein, indicating that it is not a precursor to PSCP.

\( B. \text{cepacia} \) Pc715j has more extracellular protease activity than \( B. \text{cepacia} \) K56-2. The Pc715j zmpA mutant still produces protease, suggesting that this strain produces more than one extracellular protease. In contrast, the zmpA mutant of \( B. \text{cepacia} \) K56-2 elicits minimal protease activity, suggesting that under the conditions employed in this study, PSCP may be the major extracellular protease in this strain.

Thermolysin-like metalloproteases have been implicated in bacterial pathogenesis (Morihara, 1995). In vitro assays demonstrate that PSCP is capable of cleaving biologically relevant substrates, including collagen (McKevitt et al., 1989), human IgA, IgG, IgM, transferrin and lactoferrin (C. Kooi & P. A. Sokol, unpublished observation). Previously, we have demonstrated a possible role for the extracellular zinc metalloprotease, PSCP, in \( B. \text{cepacia} \) virulence (Sokol et al., 2000). In this study, we demonstrate that the expression of a thermolysin-like protease by \( B. \text{cepacia} \)

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**Table 2. Comparison of the virulence of \( B. \text{cepacia} \) parent and zmpA mutants using a chronic respiratory infection model**

<table>
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<th>Strain</th>
<th>Virulence at p.i. day:</th>
<th>7</th>
<th>14</th>
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<tbody>
<tr>
<td></td>
<td>log(c.f.u. ml(^{-1})) per lung</td>
<td>Pathology (%)</td>
<td>log(c.f.u. ml(^{-1})) per lung*</td>
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<tr>
<td>K56-2</td>
<td>4.65 ± 0.41†</td>
<td>33.8 ± 9.0*</td>
<td>3.94 ± 1.12</td>
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<tr>
<td>K56-2-9</td>
<td>0.78 ± 1.35‡</td>
<td>9.0 ± 4.4†‡</td>
<td>0.85 ± 1.13‡</td>
</tr>
<tr>
<td>Pc715j</td>
<td>5.88 ± 0.22*</td>
<td>18.4 ± 5.4§</td>
<td>6.07 ± 0.53</td>
</tr>
<tr>
<td>Pc715j-72</td>
<td>5.89 ± 0.11*</td>
<td>24.2 ± 5.4§</td>
<td>5.83 ± 0.28</td>
</tr>
</tbody>
</table>

*Values are means ± sd for four animals.
†Values are means ± sd for three animals.
‡K56-2-9 is significantly different from K56-2 (P<0.01; Student’s unpaired t test).
§Values are means ± sd for five animals.
K56-2 contributes to virulence in an agar bead model of lung infection. The K56-2-9 zmpA mutants were less able to persist in rat lungs than the parental K56-2 strain, indicating that PSCP contributes to the persistence of \textit{B. cepacia} K56-2 within the lung. PSCP may directly degrade host tissue allowing the organisms to replicate in the lung or disrupt the host immune response by degrading immunoglobulins or other host proteins involved in the inflammatory response. This may lead to a reduced ability of the animals to clear the bacteria. The virulence of the Pc715j zmpA mutant was not decreased; however the Pc715j zmpA mutant continues to produce extracellular protease. This suggests that at least some strains of \textit{B. cepacia} produce more than one protease. Due to this possible redundancy, the loss of a single protease may not compromise the virulence of a strain in a particular infection model and, therefore, the contributions of this extracellular protease to virulence may vary among \textit{B. cepacia} strains.

Immunization with a conserved zinc metalloprotease peptide was previously shown to decrease the severity of \textit{B. cepacia} Pc715j infection. Antibodies to this peptide, however, also react with additional proteins in \textit{B. cepacia} supernatants, including a 40 kDa protein. It is possible that the reduced lung injury observed in immunized animals is due to the abilities of the induced antibodies to neutralize the proteolytic activity of PSCP as well as react with the 40 kDa protein and possibly inactivate its activity. Further studies are in progress to identify additional proteases in \textit{B. cepacia} and to determine the role of the 40 kDa protein since we have determined it is not a precursor of PSCP.

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**REFERENCES**


