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 gene 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 septicemia, 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; McKeveit & 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 (Speer 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 (McKeveit et al., 1989). McKeveit et al. (1989) demonstrated that this zinc metalloprotease is capable of cleaving gelatin, hide powder and human collagen types I, IV and V (McKeveit 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 zinc and calcium salts (McKeveit et al., 1989). PSCP may play a role in the virulence of B. cepacia. McKeveit 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/blasta_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-trypticase soy broth (Difco) treated with Chelex-100 (Bio-Rad) (Ohman *et al.*, 1980). Trypticase soy agar (TSA) (Difco) and Burkholderia cepacia 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% casetin. When required, antibiotics were used at the following concentrations: for *B. cepacia* tetracycline (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>
<thead>
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<th>Table 1. Bacterial strains and plasmids used in this study</th>
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<tr>
<td><strong>Strains or plasmid</strong></td>
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<tr>
<td><strong>E. coli</strong></td>
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<td>DH10b</td>
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<td>DH5α</td>
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<tr>
<td>TOP10</td>
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<tr>
<td><strong>B. pseudomallei</strong></td>
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<tr>
<td>1026b</td>
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<tr>
<td>K56-2</td>
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<tr>
<td>Pc715j</td>
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<tr>
<td>K56-2-9</td>
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<tr>
<td>Pc715j-72</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pCR2.1-TOPO</td>
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<td>pTOPOZMPA</td>
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<tr>
<td>pRK2013</td>
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<td>p34E-Tp</td>
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<td>pEX18Tc</td>
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<td>pCC12</td>
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<td>pCC12T</td>
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<td>pUCP26</td>
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<td>pUCP28T</td>
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<td>PUC28Tfc</td>
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<td>pCC14</td>
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Cloning zmpA from B. pseudomallei. The B. pseudomallei thermalysin sequence (accession no. X76986) was used to identify a thermolysin-like zinc metalloprotease 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). The zmpA gene from B. pseudomallei 1026b was amplified by PCR with the oligodeoxynucleotide primers NPS’ (5’-CGGGATCCGTTGCAAGTGATCTTCAAG-3’) containing a BamHI linker and NPS’ (5’-GCTCTAGATATGCTAGTGCGTT-ATCG-3’) containing an XhoI linker. The PCR products were cloned into pCR2.1-TOPO (Invitrogen Life Technologies).

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 AmpliTag DNA polymerase (Perkin-Elmer) and analysed with an ABI373A DNA sequencer 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 Biosoft). 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 CLUSTAL 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 DH5α(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 inactivation of E. coli DH10b using the API20E system (Analytab Products).

Cloning zmpA from B. cepacia K56-2. The thermolysin-like zinc metalloprotease gene (zmpA) homologue in the B. pseudomallei K96243 genome sequence at the Wellcome Trust Sanger Institute was used to identify a thermolysin-like zinc metalloprotease gene (zmpA) homologue in the B. cepacia K56-2 genome sequence using the BLASTN 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 metalloprotease 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-AAATGTGRSLYYG-230, identical to the partial N-terminal amino acid sequence described for the deduced amino acid sequence of B. cepacia zmpA (36 000 Da) observed by SDS-PAGE analysis (Kooi et al., 1994). Similar features were observed for the deduced amino acid sequence of B. cepacia zmpA cloned into pCR2.1-TOPO (Invitrogen Life Technologies).

RESULTS

Cloning and sequence analysis of the extracellular zinc metalloprotease genes

An ORF with homology to thermolysin was identified in the B. pseudomallei K96243 genome sequence using the BLASTN 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 metalloprotease 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-AAATGTGRSLYYG-230, identical to the partial N-terminal amino acid acid sequence determined sequence. 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

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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 (HEExH) 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. 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 99.5 % 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 insertional 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. 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.
and Western Immunoblots a 36 kDa protein was present in the 
\textit{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 propeptide 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 propeptide 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 \textit{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 
\textit{zmpA} mutant strains of \textit{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 
agar 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 \textit{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 \textit{zmpA} mutant, K56-2-9, which 
predicted 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

![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.](http://mic.sgmjournals.org)
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. AP120 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
Values are means ± SD for four animals.
†Values are means ± SD for three animals.
‡Values are means ± SD for five animals.

Table 2. Comparison of the virulence of B. cepacia parent and zmpA mutants using a chronic respiratory infection model

<table>
<thead>
<tr>
<th>Strain</th>
<th>Virulence at p.i. day:</th>
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<tr>
<td></td>
<td>7</td>
<td>14</td>
<td></td>
</tr>
<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>
</tr>
<tr>
<td>K56-2</td>
<td>4·65 ± 0·41†</td>
<td>33·8 ± 9·0*</td>
<td>3·94 ± 1·12</td>
</tr>
<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>
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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. 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. 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. cepacia and B. pseudomallei that encode zinc metalloproteases. The deduced amino acid sequences of the B. cepacia and B. 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. 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. 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. 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. 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. 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. cepacia zmpA mutant strains still contain the 40 kDa protein, indicating that it is not a precursor to PSCP.

B. cepacia Pc715j has more extracellular protease activity than B. 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. 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. cepacia virulence (Sokol et al., 2000). In this study, we demonstrate that the expression of a thermolysin-like protease by B. cepacia...
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 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 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 B. cepacia strains.

Immunization with a conserved zinc metalloprotease peptide was previously shown to decrease the severity of B. cepacia Pc715j infection. Antibodies to this peptide, however, also react with additional proteins in 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 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


