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Burkholderia cenocepacia ZmpB Is a Broad-Specificity Zinc Metalloprotease Involved in Virulence

C. Kooi,† B. Subsin,† R. Chen, B. Pohorelic, and P. A. Sokol*

Department of Microbiology and Infectious Diseases, University of Calgary, Calgary, Alberta, Canada T2N 4N1

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In previous studies we characterized the *Burkholderia cenocepacia* ZmpA zinc metalloprotease. In this study, we determined that *B. cenocepacia* has an additional metalloprotease, which we designated ZmpB. The *zmpB* gene is present in the same species as *zmpA* and was detected in *B. cepacia*, *B. cenocepacia*, *B. stabilis*, *B. ambifaria*, and *B. pyrrocinia* but was absent from *B. multivorans*, *B. vietnamiensis*, *B. dolosa*, and *B. anthina*. The *zmpB* gene was expressed, and ZmpB was purified from *Escherichia coli* by using the pPROEXHTa His_6 Tag expression system. ZmpB has a predicted preproenzyme structure typical of thermolysin-like proteases and is distantly related to *Bacillus cereus* bacillolysin. ZmpB was expressed as a 63-kDa preproenzyme precursor that was autocatalytically cleaved into mature ZmpB (35 kDa) and a 27-kDa prepropeptide. EDTA, 1,10-phenanthroline, and Zn^{2+} cations inhibited ZmpB enzyme activity, indicating that it is a metalloprotease. ZmpB had proteolytic activity against α-1 proteinase inhibitor, α_2-macrogobulin, type IV collagen, fibronectin, lactoferrin, transferrin, and immunoglobulins. *B. cenocepacia zmpB* and *zmpA zmpB* mutants had no proteolytic activity against casein and were less virulent in a rat agar bead chronic infection model, indicating that *zmpB* is involved in *B. cenocepacia* virulence. Expression of *zmpB* was regulated by both the CepIR and CciIR quorum-sensing systems.

The *Burkholderia cepacia* complex is comprised of nine closely related species (8, 9; reviewed in reference 39). *B. cepacia* complex organisms infect approximately 4 to 7% of cystic fibrosis (CF) patients (12). *B. cepacia* complex strains may cause a rapid deterioration of lung function and death in some CF patients (27). Strains from all nine species have been isolated from CF patients, but the most prevalent species are *B. cenocepacia* and *B. multivorans*, with strains of *B. cenocepacia* being most commonly isolated from North American CF patients (45, 55). The majority of the transmissible and epidemic *B. cepacia* complex strains belong to *B. cenocepacia* (35, 39, 41, 55). Highly transmissible *B. cenocepacia* strains, such as ET12, Midwest, and PHDC, have been identified in outbreaks in North America and Europe (6, 34). *B. cenocepacia* ET12 was responsible for the largest *B. cepacia* complex epidemic affecting CF patients in Canada and the United Kingdom during the late 1980s and early 1990s and has been linked to patient-to-patient transmission (55, 56). *B. cenocepacia* ET12 strains contain a large number of unique genes (3, 38, 56), although none of these have been directly linked to transmissibility between patients.

Numerous factors that have been implicated in *B. cenocepacia* virulence have been identified, including protease (11), quorum-sensing systems (1, 30, 54), hemolysin (26), lipopolysaccharide (25), capsule (25), cable pili and 22-kDa adhesin (49), flagella (57), exopolysaccharides (7, 10), siderophores (59), and *yfjI*, a gene of unknown function (3). We previously identified the *zmpA* gene and determined that it encodes a zinc metalloprotease that contributes to virulence in chronic lung infections (11). *B. cepacia*, *B. cenocepacia*, *B. stabilis*, *B. ambifaria*, and *B. pyrrocinia* have a *zmpA* gene and detectable extracellular protease activity, whereas *B. multivorans*, *B. vietnamiensis*, *B. dolosa*, and *B. anthina* lack *zmpA* and are protease negative (19). ZmpA has the potential to cause direct tissue damage and to modulate the host immune system, since it has been shown to degrade type IV collagen, fibronectin, α-1 proteinase inhibitor, α_2-macroglobulin, and gamma interferon (28). ZmpA is expressed as a preproenzyme that is autoproteolytically cleaved into a 36-kDa mature enzyme. It was confirmed to be a zinc metalloprotease, since its activity was inhibited by EDTA and 1,10-phenanthroline (28). Expression of the *zmpA* gene is regulated by both the CepIR and CciIR quorum-sensing systems (40, 54).

Quorum sensing is a regulatory system that controls expression of target genes in a cell density-dependent manner and in gram-negative bacteria usually involves *N*-acyl-homoserine lactones (AHLs) as signaling molecules (reviewed in references 44 and 58). AHLs are synthesized by AHL synthases encoded by *luxI* homologues. The AHLs bind to and activate a response regulator encoded by a *luxR* homologue that regulates expression of target genes at the level of transcription. Two LuxIR quorum-sensing systems, *cepIR* and *cciIR*, have been identified in *B. cenocepacia* K56-2 (1, 33, 40). CepI and CciI synthesize *N*-octanoyl-L-homoserine lactone and *N*-hexanoyl-L-homoserine lactone, although in different ratios (36, 40). The *cepIR* system is widely distributed throughout the *B. cepacia* complex (20, 36), whereas the *cciIR* system is found only in *B. cenocepacia* ET12 strains that contain the *cci* genomic island (1).

Protease activity has previously been characterized in *B. cenocepacia* strains with mutations in *zmpA* or each of the quorum-sensing genes. *B. cenocepacia* K56-2 and Pc715j *zmpA* mutants had significantly less extracellular protease activity

* Corresponding author. Mailing address: Department of Microbiology and Infectious Diseases, Faculty of Medicine, University of Calgary, Calgary, Alberta, Canada T2N 4N1. Phone: (403) 220-6037. Fax: (403) 270-2772. E-mail: psokol@ucalgary.ca.

† These authors contributed equally to this study.

than the parent strains; however, the *zmpA* mutation did not completely eliminate proteolytic activity (11). In fact, the Pc715j *zmpA* mutant retained approximately 50% of the proteolytic activity of the parent and was equally virulent in the rat agar bead lung infection model (11). *B. cenocepacia* K56-2 or H111 *cepI* or *cepR* mutants produce no detectable protease (24, 32). Interestingly, a K56-2 *cciI* mutant produced significantly more protease activity than the parent strain, although expression of a *zmpA*::*lacZ* fusion was considerably lower in the *cciI* mutant than the parent strain (40). Taken together, these studies suggested that *B. cenocepacia* has at least one extracellular protease gene in addition to *zmpA* and that there are differences between the regulation of *zmpA* and other protease genes by the *cciIR* quorum-sensing system. We isolated a spontaneous protease-negative mutant of *B. cenocepacia* K56-2 that does not express *zmpA* but has less protease activity than a *zmpA* mutant, suggesting that it was also deficient in expression of other proteases (data not shown). In the present study, we used this mutant to clone a second metalloprotease gene from *B. cenocepacia*, which we designated *zmpB*. We characterized the activity of this protease and demonstrated that it is involved in the virulence of *B. cenocepacia* and that its expression is regulated by both the CepIR and CciR quorum-sensing systems.

MATERIALS AND METHODS

Strains, plasmids, primers, and growth conditions. Strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *B. cenocepacia* were routinely grown in Luria-Bertani (LB) broth (Invitrogen, Burlington, Ontario, Canada) or on LB agar at 37°C. Bacteria from rat lung homogenates were recovered on *Burkholderia cepacia* selective agar (21). When appropriate, antibiotics were used at the following concentrations (per milliliter): for *B. cenocepacia*, 200 μg tetracycline (Tc) and 100 μg trimethoprim (Tp), and for *E. coli*, 100 μg ampicillin (Ap), 50 μg kanamycin (Km), 15 μg Tc, and 1.5 mg Tp. All chemicals were obtained from Sigma (St. Louis, Mo.) except where indicated.

DNA manipulations. Molecular biology techniques were generally performed as described by Sambrook et al. (50). PCRs were carried out using standard methods as described by the manufacturer, with either Vent DNA polymerase (New England Biolabs, Pickering, Ontario, Canada) for cloning of *zmpB* or *Taq* DNA polymerase (Invitrogen) for amplification of a *zmpB* fragment from *B. cepacia* complex strains. PCR products or DNA fragments were purified from agarose gels by using a QIAquick gel extraction kit (QIAGEN, Mississauga, Ontario, Canada). DNA sequencing was performed by the University of Calgary Core DNA Sequencing Laboratory. Sequence analysis was performed with DNAMAN software (Lynnon Biosoft, Vaudreuil, Quebec, Canada), and sequences were compared to the unpublished genome sequence of *B. cenocepacia* J2315 (http://www.sanger.ac.uk/Projects/B_cenocepacia/) by using Artemis software (48).

Cloning of *zmpB* and construction of *zmpB* mutants. *B. cenocepacia* K56-2 genomic DNA was partially digested with Sau3A and cloned into pUCP26 (60) digested with BamHI. The resultant library was electroporated into a spontaneous protease-negative mutant of K56-2 as previously described (14), and transformants were screened for restoration of proteolytic activity on brain heart infusion (BHI)-skim milk agar (53). One positive clone was designated pUCP21D6.

A 2.9-kb PCR fragment containing *zmpB* was amplified from pUCP21D6 by using primers BS1-21D6F (5′-TGTGCGTCGCGTGCGGCTTC-3′) and BS1-21D6R (5′-GTGCGCGACCTGTTCGACGG-3′) and cloned into pCR2.1-TOPO (Invitrogen), generating pBS1. For construction of pBS2 and pBS3, antibiotic cassettes (Tp^r from p34E-Tp [16] and Tc^r from p34S-Tc [15], respectively) were inserted into the pBS1 SalI site after deletion of a 1.4-kb SalI internal fragment.

A 2.1-kb XhoI-HindIII fragment from pBS2 was cloned into the suicide vector pEX18Tc and used to generate K56-2 *zmpB* by allelic exchange as previously described (22). A 2.9-kb XhoI-HindIII fragment from pBS3 was cloned into pEX18Ap (22) and transformed into *B. cenocepacia* K56-2-9 (*zmpA*) (11) to generate K56-2 *zmpA zmpB*. Allelic exchange mutants were identified by loss of the vector, sucrose resistance, and decreased zones on BHI-skim milk agar. Double-crossover mutants were confirmed by PCR using the primers pBS1-21D6F and BS1-21D6R. For complementation experiments, the 2.9-kb XhoI-HindIII fragment containing *zmpB* from pBS1 was cloned into pUCP26 (60) to generate pBS6.

Animal studies. The virulence of the K56-2 *zmpA*, K56-2 *zmpB*, and K56-2 *zmpA zmpB* mutants was compared to that of the parent K56-2 by using the agar bead chronic respiratory infection model (4). The number of bacteria and histopathological changes indicating inflammation in the infected lungs were quantified on days 7 and 14 postinfection (p.i.) as previously described (2).

For generation of anti-ZmpB sera, Sprague-Dawley rats (Charles River, Saint Constant, Quebec, Canada) were immunized on days 0 and 21 with recombinant ZmpB (approximately 20 μg) emulsified in monophosphoryl lipid A plus synthetic trehalose dicorynomycolate adjuvant as described by the manufacturer (Corixa Corporation, Hamilton, MT). Rats were bled on day 26 by cardiac puncture and anti-ZmpB antibodies detected by enzyme-linked immunosorbent assay using recombinant ZmpB as the antigen. Production of anti-ZmpA serum was previously described (19).

Expression of the *zmpB* gene and purification of recombinant ZmpB. The primers ZMPBF (5′-CGGAATTCATGCAGACATCACGCAAAGCATTG) and ZMPBR (5′-CCCAAGCTTCCTGCTGCGGTATTACGACTTCTG) were used to PCR amplify a 1,713-bp fragment containing K56-2 *zmpB* from the start codon (ATG) to 12 bp beyond the stop codon (TAA). The ZMPBF and ZMPBR primers contain an EcoRI site and a HindIII site (underlined), respectively. The amplified fragment was then digested with EcoRI-HindIII and ligated into pPROEXHTa (Invitrogen) to generate pSCM4.

E. coli DH5α(pSCM4) was grown overnight in 10 ml LB supplemented with Ap and used to inoculate 400 ml LB at an initial optical density at 600 nm (OD_{600}) of 0.03. When the cultures reached an OD_{600} of 0.6, they were induced with 0.7 mM isopropyl-β-D-thiogalactoside (IPTG) for 3.5 h. Recombinant ZmpB was purified and refolded as previously described for ZmpA (28).

The relative molecular mass and purity of ZmpB were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (31). The partial N-terminal amino acid sequence of the mature ZmpB was determined from ZmpB separated by SDS-PAGE and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Millipore Corp., Etobicoke, Ontario, Canada). Sequencing was performed by the Protein Microchemistry Laboratory at the University of British Columbia.

To detect ZmpB expression in *B. cenocepacia* and other *B. cepacia* complex species, cell-free supernatants were precipitated with trichloroacetic acid to a final concentration of 10% as previously described (11). The proteins were electrophoresed for 30 h at 80 V by Tricine-SDS-14% polyacrylamide gel electrophoresis (51) and transferred to PVDF membranes. ZmpB and ZmpA were detected on the immunoblots with rat antibodies to each protein, using peroxidase-conjugated goat anti-rat immunoglobulin G (IgG) (Kirkegaard & Perry, Gaithersburg, MD).

Protease activity assays. Protease activity was determined on BHI-skim milk agar at 24 and 48 h as previously described (28) or with hide powder azure (47). One unit of protease was defined as the activity that produced a change in the OD_{595} of 0.1 per hour at 37°C.

The optimal pH for ZmpB proteolytic activity was determined. ZmpB (4 units, 100 ng) was diluted in citrate buffer (25 mM, pH 3 to 6), morpholinoethanesulfonic acid (MES) (25 mM, pH 5 to 6.7), 3-(*N*-morpholino)propanesulfonic acid (MOPS) (25 mM, pH 6.5 to 8), Tris-HCl (25 mM, pH 8 to 9), or bicarbonate (25 mM, pH 10) and incubated at 37°C for 2 h. The optimum temperature for ZmpB activity was determined in 25 mM MES (pH 5.6). Assays were performed in triplicate with a buffer control at each temperature, using hide powder azure as the substrate.

To identify potential ZmpB substrates, ZmpB (2 units) was incubated for 24 to 48 h at 37°C with α-1 proteinase inhibitor (Bayer Corporation, Elkhart, IN), human fibronectin, type IV collagen, lactoferrin, transferrin, IgA, IgG, IgM, rat α_2-macroglobulin, and recombinant rat gamma interferon (Sigma) in 25 mM MES, pH 5.6. Recombinant ZmpB and substrate-only controls were included. In addition, substrates were incubated with the proteolytically inactive ZmpA H465G (28) purified in an identical manner as ZmpB to ensure that proteolytic activity was not due to contaminating proteases in the reaction mixtures.

For inhibitor and divalent metal cation studies, ZmpB (4 units, approximately 100 ng) was preincubated with the specified inhibitor(s) and/or divalent metal cation(s) for 30 min at room temperature and transferred to hide powder azure in 25 mM MES (pH 5.6). Neutralization assays with a monoclonal antibody (36-6-8) to ZmpA were performed as previously described (29).

Expression of *zmpB*::*luxCDABE* transcriptional fusions. A *zmpB*::*luxCDABE* transcriptional fusion was constructed in pMS402 (17). A 0.75-kb DNA fragment containing the *zmpB* promoter was PCR amplified from K56-2 genomic DNA

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description or origin[a]	Reference or source
Strains		
E. coli		
DH5α	F$^-$ ϕ80d*lacZ*Δ*M15* Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44 thi-1 gyrA96 relA1*	Invitrogen
TOP10	F$^-$ *mcrA* Δ(*mrr-hsd*RMS*mcrBC*) ϕ80*lac* Δ*M15* Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)7697 *galU galK rpsL*(Sm^r) *endA1 nupG*	Invitrogen
B. cepacia		
ATCC 25416	Onion, United States	37
ATCC 17759	Soil, Trinidad	37
Cep 509	CF, Australia	37
LMG17997	UTI, Sweden	37
B. multivorans		
C5393	CF, Canada	37
LMG13010	CF, Belgium	37
C1576	CF-e, United Kingdom	37
CF-A1-1	CF-e, United Kingdom	37
JTC	CGD, United States	37
C1962	Clinical, United Kingdom	37
ATCC 17616	Soil, United States	
249-2	Laboratory, United States	37
B. cenocepacia		
J2315	CF-e, United Kingdom	37
BC7	CF-e, Canada	37
K56-2	CF-e, Canada	37
C5424	CF-e, Canada	37
C6433	CF-e, Canada	37
C1394	CF-e, United Kingdom	37
PC184	CF-e, United States	37
CEP511	CF-e, Australia	37
J415	CF, United Kingdom	37
ATCC 17765	UTI, United Kingdom	37
PC715j	CF, Canada	37
K562-9	K56-2 *zmpA*::*tp*; Tp^r	12
K56-2 *zmpB*	K56-2 *zmpB*::*tp*; Tp^r	This study
K56-2 *zmpA zmpB*	K56-2 *zmpA*::Tp^r and *zmpB*::tc; Tp^r, Tc^r	This study
K56-dI2	Δ*cepI* derivative of K56-2	40
K56-R2	*cepR*::Tn*5*-OT182 derivative of K56-2	32
K56-2 *cciI*	*cciI*::Tp derivative of K56-2	1
K56-2 *cciR*	*cciR*::Tp derivative of K56-2	40
K56-2 *cciIR*	ΔP*cciIR*::Tp derivative of K56-2	40
K56-2 *cepR cciIR*	*cepR*::Tp ΔP*cciIR* derivative of K56-2	40
B. stabilis		
LMG 14294	CF, Belgium	37
C7322	CF, Canada	37
LMG 14086	Respirator, United Kingdom	37
LMG 18888	Clinical, Belgium	37
B. vietnamiensis		
PC 259	CF, United States	37
LMG 16232	CF, Sweden	37
FC 441	CGD, Canada	37
LMG 10929^T	Rice, Vietnam	37
B. dolosa		
LO6	CF	9
LMG 18943	CF, United States	9
LMG 19468t		9
B. ambifaria		
ATCC 53266	Corn roots, biocontrol, United States	9
CEP0996	CF, Australia	9
B. anthina		
W92^T	Soil, United States	9

Continued on following page

TABLE 1—*Continued*

Strain or plasmid	Description or origin[a]	Reference or source
C1765	CF, United Kingdom	9
J2552	Rhizosphere, United Kingdom	9
LMG 20982		D. P. Speert
B. pyrrocinia		
ATCC 15958^T	Soil, Japan	9
ATCC 39277	Soil, United States	9
Plasmids		
pCR2.1-TOPO	TOPO-TA cloning vector; Ap^r Km^r	Invitrogen Life Technologies
pEX18Tc	Suicide vector; *sacB* Tc^r	22
pUCP26	Broad-host-range vector; Tc^r	60
pRK2013	ColE1 tra (RK2)$^+$; Km^r	18
p34E-Tp	Source of trimethoprim cassette	16
p34S-Tc	Source of tetracycline cassette	15
pUCP21D6	pUCP26 with 5.9-kb insert containing *zmpB*; Tc^r	This study
pMS402	*lux*-based promoter reporter plasmid; Km^r Tp^r	17
pBS1	pCR2.1-TOPO with 2.9-kb PCR fragment containing the *zmpB* gene; Ap^r Km^r	This study
pBS2	pBS1 with 1.4-kb SalI deletion in *zmpB* and insertion of Tp^r; *zmpB*Δ::Tp^r	This study
pBS3	pBS1 with 1.4-kb SalI deletion in *zmpB* and insertion of Tc^r; *zmpB*Δ::Tc^r	This study
pBS6	pUCP26 with 2.9-kb XbaI-HindIII fragment from pBS1 containing *zmpB*; Tc^r	This study
pBS8	*zmpB*::*luxCDABE* transcriptional fusion in pMS402; Km^r Tp^r	This study
pBS9	pBS8 with tetracycline cassette from p34S-Tc; Km^r Tp^r Tc^r	This study
pPROEX-HTa	*E. coli* expression vector with His_6 tag, TEV protease cleavage site, and inducible *trc* promoter; Ap^r	Invitrogen
pSCM4	pPROEX-Hta with 1.7-kb fragment containing *zmpB*; Ap^r	This study

[a] UTI, urinary tract infection; CF-e, epidemic strain from CF patients; CGD, chronic granulomatous disease.

with BSpzmpBF (5′-ATACTTTCGTATCGGCCAC-3′) and BSpzmpBR (5′-ACG CACTTCTACCTGAACCT-3′). The amplified fragment was digested with BamHI-XhoI and ligated into the BamHI-XhoI site upstream of *luxCDABE* in pMS402, resulting in pBS8. To generate pBS9, a SacI fragment from p34STc (15) containing the tetracycline resistance cassette was ligated into pBS8 digested with XhoI.

Luminescence assays were performed to measure expression of *zmpB*. Overnight cultures were subcultured in triplicate to an initial OD_{600} of 0.02 in 20 ml LB and grown at 37°C and 250 rpm. At 16 and 20 h, 150-μl aliquots were transferred into 96-well clear-bottom black plates (9520 Costar; Corning Incorporated, Corning, NY), and the luminescence in counts per second and turbidity at an absorbance of 600 nm were measured using a Wallac Victor^2 1420 multilabel counter (Perkin-Elmer Life Sciences, Boston, Mass.). Expression levels of *zmpB* were determined by relative luminescence.

Distribution of the *zmpB* gene in the *B. cepacia* complex. To determine the distribution of the *zmpB* gene in *Burkholderia* species, primers ZPBDTF (GCA GACATCACGCAAAGCATTG) and ZPBDTR (GCAGCTCTTGTTGTACG CGTTG) were used to PCR amplify a 1.7-kb *zmpB* fragment. The amplification conditions were 95°C for 7 min; followed by 30 cycles of 95°C for 1 min, 56°C for 30 s, and 72°C for 2 min; followed by 72°C for 20 min.

Southern hybridization analysis was also performed on genomic DNA isolated from representative strains digested with NotI. A 0.8-kb MluI fragment encoding from Arg-35 to Asn-307 of the 323-amino-acid mature ZmpB was labeled with ^{32}P by random priming as described by the manufacturer (GE Healthcare) and used as a probe. Blots were hybridized with the probe at 65°C and washed at 72°C.

RESULTS

Identification of the *zmpB* gene. The plasmid pUCP21D6 with a 6,780-bp insert containing a putative metalloprotease gene was identified by complementation of a spontaneous protease-negative mutant that does not express *zmpA* (data not shown) with a *B. cenocepacia* K56-2 genomic library. Analysis of this insert by using the unpublished annotation of *B. cenocepacia* J2315 at the Sanger Institute (http://www.sanger.ac.uk/Projects/B_cenocepacia/) and Artemis software (48) revealed five open reading frames encoding two transcriptional regulators of the AraC family (BCAM2305 and BCAM2306), a thermolysin metalloprotease (BCAM2307), a leucyl aminopeptidase (BCAM2308), and a hypothetical protein (BCAM2309). BCAM2307, encoding a putative thermolysin-like metalloprotease belonging to the M4 family of peptidases, was designated *zmpB*, and its corresponding protein was designated ZmpB. Alignment analysis of ZmpB with the proteases most related to it, bacillolysin from *Bacillus cereus* ATCC 14579 (29% identity) and thermolysin peptidase M4 from *Bacillus anthracis* A2012 (27% identity), indicated that the HExxH active-site motif and amino acids downstream of the active site (including GxxxExxxD, where E is a putative zinc ligand) are conserved. Functional domain analysis using the Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd) demonstrated that ZmpB is also functionally related to *Pseudomonas aeruginosa* LasB and has a prepropeptide structure typical of secreted metalloproteases. The precursor to ZmpB has a theoretical pI of 5.84 and a molecular mass of 60547 Da (http://us.expasy.org.). The pre sequence has a hydrophobic transmembrane domain at amino acids 7 to 26 (http://www.sbc.su.se/~miklos/DAS) and a predicted SignalP recognition cleavage site 24TVA. The N-terminal amino acid sequence (FVFRPDPLS) was determined with a recombinant ZmpB peptide described below. Based on this amino acid sequence, the mature ZmpB has a predicted molecular mass of 34.9 kDa and pI of 5.08.

Characterization of K56-2 *zmpB* and K56-2 *zmpA zmpB* mutants. Mutation of the *zmpB* gene in K56-2 and K56-2-9 *zmpA*

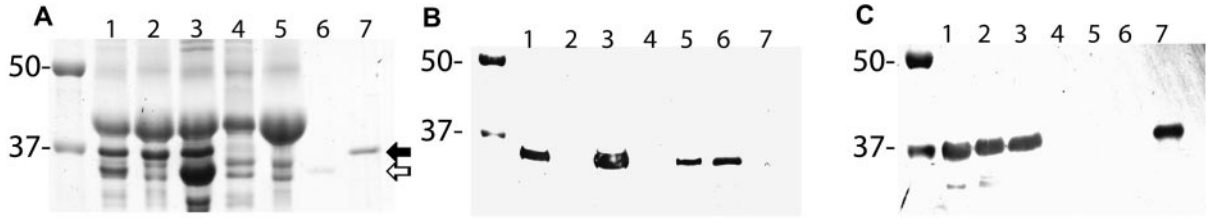

FIG. 1. Expression of ZmpA and ZmpB in K56-2 *zmpB* and K56-2 *zmpA zmpB* mutants. Supernatant proteins were precipitated with 10% trichloroacetic acid, separated by Tricine–14% SDS-PAGE, and blotted to PVDF membranes. A. Coomassie blue-stained gel. B. Western blot reacted with anti-ZmpB. C. Western blot reacted with anti-ZmpA. Lanes 1, K56-2; lanes 2, K56-2 *zmpB*; lanes 3, K56-2 *zmpB*(pBS6); lanes 4, K56-2 *zmpA zmpB*; lanes 5, K56-2 *zmpA*; lanes 6, recombinant ZmpB; lanes 7, recombinant ZmpA. Solid arrow, ZmpA; open arrow, ZmpB.

was performed by insertional inactivation of *zmpB* with a Tp^r or Tc^r cassette, resulting in K56-2 *zmpB* and K56-2 *zmpA zmpB*, respectively. The extracellular protein profiles were analyzed by Tricine-SDS-14% PAGE (Fig. 1). K56-2 and K56-2-9 *zmpA* expressed a 35-kDa protein, which corresponded to the predicted molecular mass of ZmpB. The 35-kDa protein was not detected in K56-2 *zmpB* and K56-2 *zmpA zmpB* supernatants (Fig. 1A). The 35-kDa proteins of K56-2, K56-2-9 *zmpA*, and K56-2 *zmpB*(pBS6) reacted with anti-rat ZmpB antibody (Fig. 1B), indicating that pBS6 complemented K56-2 *zmpB*. The 36-kDa peptides present in K56-2, K56-2 *zmpB*, and K56-2 *zmpB* (pBS6) correspond to the molecular mass of ZmpA (29, 42) and reacted with anti-ZmpA (Fig. 1C). Anti-ZmpA and anti-ZmpB antibodies did not cross-react with the heterologous proteases, suggesting that these two proteases are antigenically distinct.

The proteolytic activities of K56-2, K56-2 *zmpB*, and K56-2 *zmpA zmpB* mutants were determined on BHI-skim milk agar (Table 2). K56-2 had significantly more protease activity than K56-2 *zmpB* and K56-2 *zmpA zmpB*. K56-2 *zmpB*(pBS6) had zones comparable in size to those of K56-2, indicating that pBS6, containing *zmpB*, complemented the protease-negative phenotype of K56-2 *zmpB*. The protease activity of K56-2-9 *zmpA* was similar to that previously reported (11). These results indicate that *zmpB* is responsible for the majority of the protease activity of *B. cenocepacia* K56-2. Complementation of K56-2 *zmpB* with pBS6 did not result in increased protease activity above that of the wild type despite the potential for increased amounts of ZmpB protein due to the multicopy plasmid. Similar results were reported for complementation of *zmpA* mutants (11).

The effect of a *zmpB* mutation on virulence was examined using a chronic respiratory infection model. Previously, we reported that the K56-2-9 *zmpA* mutant was less persistent in lungs and manifested less histopathology than the parent K56-2 (11). Similar results were obtained in this study in that the number of K56-2-9 *zmpA* or K56-2 *zmpA zmpB* organisms recovered from the lungs at day 14 p.i. was approximately three log units lower than the number of K56-2 organisms recovered (Table 3). Three of the four rats infected with K56-2-9 *zmpA* were completely cleared of bacteria by day 14 p.i. Bacteria recovered from the fourth rat were determined by sequence analysis to be revertants. The numbers of K56-2 *zmpB* organisms recovered from the lungs were similar to those of K56-2 at both 7 and 14 days p.i. (Table 3), suggesting that *zmpA* plays a role in bacterial persistence but that *zmpB* does not.

Both *zmpA* and *zmpB* contribute to lung pathology. The lungs from the rats infected with the K56-2 *zmpA*, K56-2 *zmpB*, or K56-2 *zmpA zmpB* protease mutant had significantly fewer histopathological changes than the lungs from rats infected with the K56-2 parent (Table 3), indicating that both proteases contribute to the maximal virulence of *B. cenocepacia* K56-2 in this infection model. There was no significant difference in the quantitative histopathological changes in the K56-2 *zmpA zmpB* double mutant and the *zmpA* or *zmpB* single mutant.

Purification and characteristics of recombinant ZmpB. The *zmpB* gene was cloned into pPROEXHTa to construct pSCM4 and was expressed as an N-terminal His-tagged protein. *E. coli* DH5α(pSCM4) was proteolytically active on BHI-skim milk agar, with zones of approximately 2 to 4 mm after 48 h, in contrast to *E. coli* DH5α(pPROEXHTa), which displayed no proteolytic activity. The *zmpB* gene was expressed as a 63-kDa protein in the cell lysate after IPTG induction for 3.5 h (Fig. 2, lane 1). The mass of 63 kDa corresponded to the predicted

TABLE 2. Protease activities of *B. cenocepacia* K56-2 *zmpB* and *zmpA* mutants

Strain	Zone (mm)[a] at:	
	24 h	48 h
K56-2	3.3 ± 0.3	6.2 ± 0.3
K56-2 *zmpB*	0.0 ± 0.0*	0.0 ± 0.0*
K56-2 *zmpA zmpB*	0.0 ± 0.0*	0.0 ± 0.0*
K56-2 *zmpB*(pUCP26T)	0.0 ± 0.0*	0.0 ± 0.0*
K56-2 *zmpB*(pBS6)	3.6 ± 0.1	6.7 ± 0.3
K56-2-9 *zmpA*	0.6 ± 0.1*	1.3 ± 0.3*

[a] Values are the mean zone of clearing ± standard deviation for three replicates and are representative of at least two experiments. *, significantly different from value for K56-2 ($P < 0.05$ by analysis of variance).

TABLE 3. Virulence of K56-2 *zmpA* and *zmpB* mutants in a chronic respiratory infection model

Strain	Inoculum (CFU)	Virulence[a] at day p.i.:			
		7		14	
		Log CFU ml^{-1}	Inflammation (%)	Log CFU ml^{-1}	Inflammation (%)
K56-2	4.4×10^4	6.8 ± 7.0	49.7 ± 6.0	5.7 ± 5.4	66.8 ± 6.3
K56-2 *zmpA*	9.8×10^4	ND	ND	2.5 ± 2.7	10.3 ± 1.7*
K56-2 *zmpB*	3.2×10^4	4.5 ± 4.5	22.8 ± 3.4*	5.0 ± 5.0	16.2 ± 1.9*
K56-2 *zmpAB*	6.2×10^4	0.9 ± 0.9	12.0 ± 1.8*	2.1 ± 2.4	10.6 ± 1.1*

[a] Values are means ± standard deviations for four or five rats per group. *, significantly different from value for K56-2 at the same day p.i. ($P < 0.05$ by analysis of variance). ND, not determined.

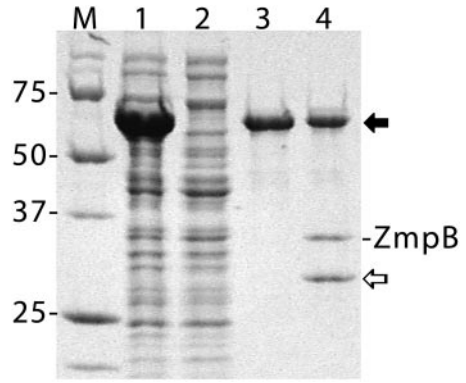

FIG. 2. Expression and purification of ZmpB. Lane M, molecular weight markers (in thousands); lane 1, cell lysate after induction with IPTG; lane 2, uninduced crude cell extract; lane 3, purified inclusion bodies; lane 4, purified recombinant ZmpB after refolding and elution from an Ni-NTA column. Solid arrow, His-prepro-ZmpB fusion protein; open arrow, indicates the His-prepropeptide. The mature ZmpB is indicated.

molecular mass of the His_6-tagged ZmpB (63.4 kDa). When the cell lysate was fractionated and analyzed by SDS-PAGE, the 63-kDa protein was recovered almost exclusively in the precipitate as insoluble inclusion bodies (Fig. 2, lane 3). When the inclusion bodies were solubilized in 6 M guanidine-HCl and applied to an Ni-nitrilotriacetic acid (NTA) affinity column to purify and refold the 63-kDa protein, two new peptides (35 kDa and 28 kDa) were identified in the eluant (Fig. 2, lane 4). The N-terminal sequence of the 35-kDa peptide was determined to be FVFRPDPLS, which is present in the C-terminal portion of the 63-kDa His_6-tagged ZmpB, and this mass corresponds to the predicted mass of 34.9 kDa that would be obtained for a peptide with this N-terminal sequence. Therefore, the 35-kDa peptide contains the active site and is the mature protease. The 28-kDa peptide has the predicted mass for the prepropeptide and the His tag.

ZmpB eluted from the Ni-NTA column was proteolytically active against hide powder azure, with 50 ng containing approximately 5 U of activity. The effect of protease inhibitors or divalent cations on ZmpB protease activity was examined. ZmpB activity was inhibited by EDTA and 1,10-phenanthroline, which chelate Zn^{2+}, but not by phenylmethylsulfonyl fluoride or phosphoramidon (Table 4). ZmpB was not inhibited by 1,7-phenanthroline, an isomer of 1,10-phenanthroline that does not bind Zn^{2+}. In addition, 1 mM Zn^{2+} abrogated ZmpB activity, but 1 mM $CaCl_2$ had no effect (Table 4). Co^{2+} and Ni^{2+} also significantly reduced ZmpB activity. Monoclonal antibody 36-6-8, which previously was shown to inhibit ZmpA, did not inhibit ZmpB activity (29). The A_{595} when ZmpB was preincubated with monoclonal antibody 36-6-8 was 0.7 ± 0.2, compared with 0.7 ± 0.1 in control ascites fluid.

The optimal pH range for ZmpB activity was between pH 5.6 and 7.2, depending on the buffers employed (data not shown). ZmpB was active over a broad pH range, although activity was dramatically decreased at pH values below 4.5 or above 8.0. ZmpB was active between 28 and 60°C, with an optimum temperature of 50°C (data not shown). Activity was significantly decreased at temperatures above 70°C.

ZmpB activity against host proteins. The ability of ZmpB to degrade a number of biologically important substrates which might account for its role in virulence was determined by incubating ZmpB with the substrate and determining proteolysis by SDS-PAGE (Fig. 3). ZmpB was able to digest the human plasma protease inhibitors α-1 proteinase inhibitor and α_2-macroglobulin. ZmpB also digested type IV collagen, fibronectin, transferrin, lactoferrin, and immunoglobulins. Collagen, fibronectin, α-1 proteinase inhibitor, and α_2-macroglobulin were cleaved into numerous peptides, indicating that ZmpB has broad specificity. Transferrin, lactoferrin, and the immunoglobulins IgA, IgG, and IgM were also cleaved into several peptides but were less extensively degraded by ZmpB than collagen, fibronectin, α-1 proteinase inhibitor, and α_2-macroglobulin.

In order to ensure that the ZmpB activity was not due to the presence of a contaminating *E. coli* protease, these substrates

TABLE 4. Effect of protease inhibitors and divalent cations on ZmpB activity

Addition to reaction mixture	Activity (units)[a]	% of control activity
None	5.0 ± 0.1	100
EDTA		
1 mM	0.2 ± 0.04*	4.0
5 mM	0.4 ± 0.02*	8.0
10 mM	0.4 ± 0.03*	8.0
1,10-Phenanthroline		
1 mM	0.8 ± 0.1*	16.0
5 mM	0.8 ± 0.1*	16.0
10 mM	0.8 ± 0.04*	16.0
1,7-Phenanthroline, 10 mM	5.4 ± 0.09	108
Phenylmethylsulfonyl fluoride		
0.1 mM	5.4 ± 0.22	108
0.5 mM	4.9 ± 0.16	98
1.0 mM	4.4 ± 0.11	88
Phosphoramidon		
1 μM	5.9 ± 0.06	118
5 μM	6.0 ± 0.15	120
10 μM	6.1 ± 0.17	122
$CaCl_2$, 1 mM	4.7 ± 0.24	94
$ZnCl_2$, 1 mM	0 ± 0.02*	0
$ZnSO_4$, 1 mM	0 ± 0.02*	0
$CoCl_2$, 1 mM	1.2 ± 0.03*	24
$MgCl_2$, 1 mM	3.6 ± 0.01	72
$MgSO_4$, 1 mM	4.1 ± 0.08	82
$MnCl_2$, 1 mM	2.5 ± 0.05*	50
$MnSO_4$, 1 mM	3.4 ± 0.07	68
$NiSO_4$, 1 mM	0.3 ± 0.02*	60

[a] Proteolytic activity was determined using hide powder azure as the substrate. Values are means ± standard deviations *, significantly different from the control value ($P < 0.01$ by analysis of variance and Dunnett multiple-comparisons test).

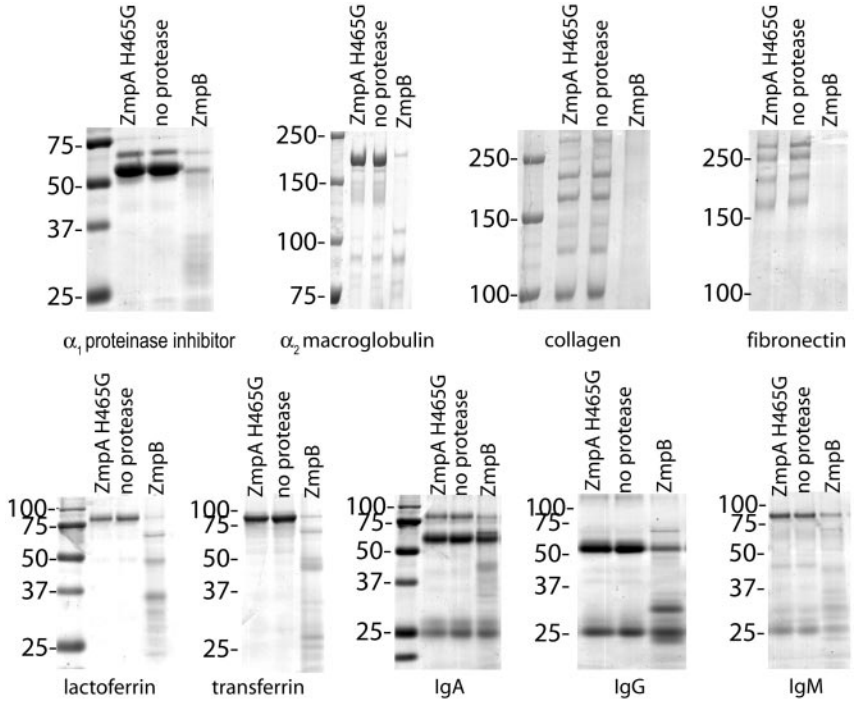

FIG. 3. Substrate specificity of ZmpB. Substrates were digested with ZmpB, ZmpAH465G (which has no proteolytic activity), or in the absence of enzyme (no protease) for 24 to 48 h. Proteins were separated by SDS-PAGE and stained with Coomassie blue. Collagen, fibronectin, and α_2-macroglobulin were electrophoresed by SDS-7.5% PAGE. All other substrates were electrophoresed by SDS-12.5% PAGE.

were digested with a recombinant inactive form of ZmpA, ZmpA H465G (28), that was purified using the same protocol. ZmpA H465G had no activity against any of the substrates, confirming that the cleavage of all substrates was not due to the presence of contaminating proteases in the reaction mixtures or proteases that might copurify from *E. coli*.

Quorum-sensing regulation of *zmpB* expression. Previously, *zmpA* had been shown to be regulated by the *cepIR* and *cciIR* quorum-sensing systems (40, 54). To determine if *zmpB* expression was regulated by *cepIR* or *cciIR*, the *zmpB* promoter region was cloned upstream of the *luxCDABE* operon in pMS402 (17). The resulting plasmid, pBS9, was transformed into K56-2, K56-dI2 *cepI*, K56-R2 *cepR*, K56-2 *cciI*, K56-2 *cciR*, K56 *cciIR*, and K56-2 *cepR cciIR*. Expression of *zmpB*::*luxCDABE* was reduced to almost background levels in K56-dI2(pBS9) and K56-R2(pBS9) and was significantly reduced in K56-2 *cciR* (pBS9) and K56-2 *cepR cciIR*(pBS9) (Fig. 4). Interestingly, *zmpB*::*luxCDABE* was significantly increased in K56-2 *cciI* (pBS9). Expression in K56-2 *cciIR*(pBS9) was similar to that in the parent. These data suggest that *zmpB* expression is regulated by both *cepIR* and *cciIR*.

Distribution of the *zmpB* gene in *Burkholderia* spp. To investigate the distribution of the *zmpB* gene in *Burkholderia* species, primers ZPBDTF and ZPBDTR were used to PCR amplify the 1.7-kb *zmpB* gene in 43 strains representing the *B. cepacia* complex. A 1.7-kb amplicon was obtained from all strains of *B. cepacia*, *B. cenocepacia*, *B. ambifaria*, and *B. pyrrocinia* examined but not from strains of *B. multivorans*, *B. vietnamiensis*, *B. dolosa*, and *B. anthina* (data not shown). Southern blot hybridization analysis was also performed to determine which species contained a *zmpB* gene (Fig. 5A). *B.*

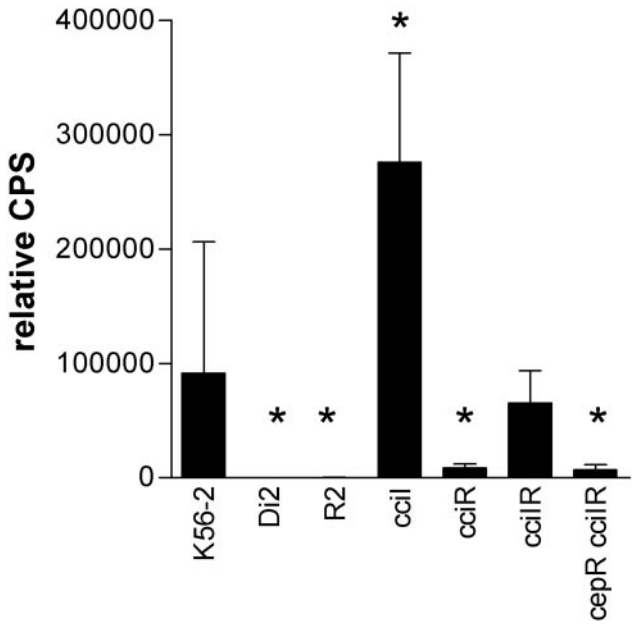

FIG. 4. Regulation of *zmpB* by the *cepIR* and *cciIR* quorum-sensing genes. Plasmid pBS9 containing a *zmpB*::*luxCDABE* fusion was introduced into K56-2 quorum-sensing mutants. Cultures were grown for 20 h, and luminescence in counts per second (CPS) and turbidity at an absorbance of 600 nm were measured using a Wallac Victor^2 1420 multilabel counter. Values shown are the mean relative CPS (±standard deviation) for triplicate cultures and are representative of three independent experiments. *, significantly different from result for K56-2 ($P < 0.05$ by analysis of variance).

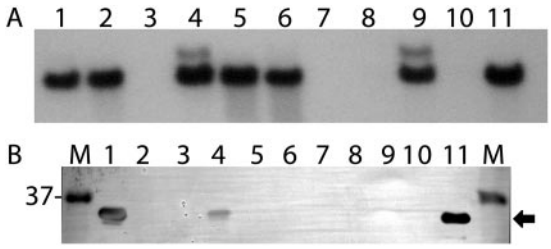

FIG. 5. Distribution of ZmpB in the *B. cepacia* complex. A. Southern hybridization analysis of the *zmpB* gene. A 0.8-kb ^{32}P-labeled MluI fragment encoding from Arg-35 to Asn-307 of the 323-amino-acid mature ZmpB from K56-2 was used to probe NotI-digested genomic DNA. Lane 1, K56-2; lane 2, *B. cepacia* LMG17997; lane 3, *B. multivorans* LMG13010; lane 4, *B. cenocepacia* J2315; lane 5, *B. stabilis* LMG14294; lane 6, *B. stabilis* C7322; lane 7, *B. vietnamiensis* PC259; lane 8, *B. dolosa* LMG18943; lane 9, *B. ambifaria* Cep996; lane 10, *B. anthina* LMG20980; lane 11, *B. pyrrocinia* LMG21822. B. Detection of ZmpB by Western blotting of culture supernatants. Trichloroacetic acid-precipitated culture supernatants were separated by Tricine 14%-PAGE and blotted onto PVDF membrane. Blots were reacted with anti-ZmpB. Lane 1, K56-2; lane 2, *B. cepacia* LMG17997; lane 3, *B. multivorans* LMG13010; lane 4, *B. cenocepacia* C6433; lane 5, *B. stabilis* LMG18888; lane 6, *B. vietnamiensis* Pc259; lane 7, *B. dolosa* LMG18943; lane 8, *B. ambifaria* CEP996; lane 9, *B. anthina* LMG20980; lane 10, *B. pyrrocinia* LMG 21822; lane 11, recombinant ZmpB.

cepacia, *B. cenocepacia*, *B. stabilis*, *B. ambifaria*, and *B. pyrrocinia* had a 0.8-kb NotI band that hybridized to the *zmpB* probe as strongly as that of the positive control strain K56-2 (Fig. 5A). The probe did not hybridize to *B. multivorans*, *B. vietnamiensis*, *B. dolosa*, or *B. anthina*, suggesting that these species do not contain a gene related to *zmpB*, although it is possible that these species could contain a related gene with less homology than would be detected by Southern hybridization.

To determine if the presence of *zmpB* correlated with extracellular protease activity, we examined the activity of 43 strains on BHI-skim milk agar. The majority (18 out of 23) of the *zmpB*-positive strains had extracellular protease activity, while none of the *zmpB*-negative strains produced detectable extracellular protease activity. These data suggest that the strains in which *zmpB* was not detectable by PCR or Southern hybridization do not contain a related gene. ZmpB was easily detected only in *B. cenocepacia* concentrated culture supernatants by Western blot analysis (Fig. 5B). Possibly ZmpB was not expressed and/or secreted in the other *zmpB*-positive *B. cepacia* complex strains under the conditions employed, or ZmpB was secreted but the anti-ZmpB polyclonal antibody employed did not react with the ZmpB from these strains.

DISCUSSION

Metalloproteases are widely distributed in nature and belong to four superfamilies or clans. All bacterial zinc metalloproteases belong to the zincins superfamily, which contains a conserved HEXXH motif. The two histidines are zinc ligands, and the glutamic acid is involved in enzymatic activity. The zincins can be subdivided, based on the position of the third zinc ligand (43), into at least 10 families, with 3 of the families containing bacterial metalloproteases: thermolysin, serralysin, and neurotoxins.

B. cenocepacia ZmpB is a novel thermolysin-like protease that has only 28% identity with its closest homologue, bacillolysin from *Bacillus cereus*, and shares only 10.3% identity with *B. cenocepacia* ZmpA. ZmpB has a preproenzyme structure that is typical of the M4 family (http://merops.sanger.ac.uk/) of metalloproteases, with conservation of functional regions such as the HExxH active-site motif as well as other conserved amino acids downstream of the active site, including Gxxx ExxxD. De Kreij et al. (13) found that there was a great deal of sequence variability among various thermolysin-like proteases but that as long as a small subset of amino acids was conserved (e.g., active site residues and zinc ligands), the group was extremely homogeneous in terms of catalysis. *B. cenocepacia* ZmpB is inhibited by EDTA and 1,10-phenanthroline, indicating that it is a metalloprotease. ZmpB is thermostable and active over a broad neutral pH range, similar to other bacterial thermolysin-like metalloproteases. ZmpB activity was relatively unaffected by Ca^{2+} and Mg^{2+} but completely abrogated by excess Zn^{2+}, as has been reported with thermolysin (23). Co^{2+} and to lesser degree Mn^{2+} could substitute for Zn^{2+} in thermolysin (23), which is also suggested by our findings for ZmpB. Ni^{2+} has not been shown to inhibit thermolysin, but Ni^{2+}, Zn^{2+}, and Cu^{2+} have been shown to inhibit a *Vibrio vulnificus* broad-specificity metalloprotease (5). Interestingly, ZmpB was not inhibited by phosphoramidon, a peptide inhibitor of metalloproteases that is related to *B. thermoproteolyticus* thermolysin. *B. cenocepacia* ZmpA is also unaffected by phosphoramidon (28). Phosphoramidon is a peptide that binds to a specific sequence in the thermolysin active-site region. Sequence differences in this region have been correlated with reduced phosphoramidon sensitivity (13).

ZmpB was shown to be a broad-spectrum protease capable of degrading substrates involved in tissue integrity and host defense. Most proteases are inhibited by α_2-macroglobulin, but both ZmpB and ZmpA (28) are able to digest α_2-macroglobulin and α-1 proteinase inhibitor, indicating that *B. cenocepacia* produces two extracellular proteases that can inactivate the major mammalian protease inhibitors. Inactivation of α-1 proteinase inhibitor in vivo may disrupt the α-1 proteinase/α-1 proteinase inhibitor balance and lead to increased tissue damage and inflammation. Inactivation of α_2-macroglobulin could result in increased *B. cenocepacia* dissemination and lead to septicemia. Both ZmpB and ZmpA can degrade host tissue components, including collagen and fibronectin. Although ZmpB and ZmpA have activity against many of the same substrates, each enzyme has a different specificity. ZmpA cleaved α-1 proteinase inhibitor specifically at D_{36} (D_{36}TSHHDQD) (28), whereas ZmpB cleaved α-1 proteinase inhibitor into a number of fragments (Fig. 3). ZmpA specifically cleaved α_2-macroglobulin into major polypeptides of 95 and 86 kDa (28). ZmpB cleaved α_2-macroglobulin into numerous peptides (Fig. 3).

ZmpB was shown to cleave immunoglobulins (IgA, IgG, and IgM), transferrin, and lactoferrin, which could also increase the ability of *B. cenocepacia* to evade host defenses. Although we previously cited unpublished data (52) that suggested that ZmpA had activity against these substrates, recombinant and native ZmpA were subsequently shown not to degrade these substrates (28). It is likely that the protease preparations used in the unpublished study contained a mixture of both ZmpA and ZmpB proteases. ZmpB digested lactoferrin, transferrin,

and immunoglobulins into markedly fewer peptides than α_2-macroglobulin, collagen, and fibronectin, suggesting that there is some cleavage site specificity. ZmpA was shown to digest gamma interferon (28). Because ZmpB had significant proteolytic activity against the albumin present in large amounts in the gamma interferon preparation used, it was not possible to conclusively distinguish between gamma interferon and albumin cleavage products in this assay.

ZmpA was previously shown to be necessary for *B. cenocepacia* K56-2 to cause persistent respiratory infections in rats (11). Similar results were obtained in the present study, in that the numbers of K56-2 *zmpA* and K56-2 *zmpA zmpB* organisms recovered from infected lungs were approximately 3 log units lower than in the parent strain. In contrast, K56-2 *zmpB* was able to survive at parental levels in the rat lung, indicating that ZmpB is not required for persistence in this strain. Infection with any of the mutants resulted in significantly less lung inflammation than infection with the parent strain. There was no significant difference between the quantitative histopathology changes in lungs infected with the mutants. The decreased inflammation observed in lungs infected with *zmpA* mutants compared to the parent is at least partially due to the decreased numbers of bacteria present in the lung, whereas the decreased inflammation observed with the *zmpB* mutant is likely due to the absence of this enzyme. Although a *zmpA* mutation in K56-2 resulted in decreased virulence, a Pc715j *zmpA* mutant was virulent in the lung infection model (11). Unfortunately, we were not able to construct a *zmpB* mutation in either Pc715j or Pc715j *zmpA* to determine its effects on virulence.

Although *zmpA* and *zmpB* are not genetically linked and are in fact located on different chromosomes, they are present in the same *B. cepacia* complex species. Both genes are present in *B. cepacia*, *B. cenocepacia*, *B. stabilis*, *B. ambifaria*, and *B. pyrrocinia* but are absent from *B. multivorans*, *B. vietnamiensis*, *B. dolosa*, and *B. anthina* (19). Presence or absence of these genes is conserved within each species. Highly (>95%) conserved ZmpB homologues are present in the genomes of *B. cepacia* R18194, *B. cenocepacia* J2315, *B. cenocepacia* HI2424, and *B. ambifaria* AMMD.

Some *B. cepacia* complex strains were previously reported to have no detectable protease activity despite having the *zmpA* gene (19). Others had similar levels of protease activity, but the induction of protease production appeared to be much slower. Interestingly, all of these *zmpA*-positive, protease-deficient strains are also *zmpB* positive, suggesting that *zmpB* may also be poorly expressed or nonfunctional in these strains. These data suggest that *zmpA* and *zmpB* share similar regulatory mechanisms. It is also possible that these enzymes act synergistically, since they have different cleavage properties. The *zmpB* mutant has no activity against casein in the BHI-skim milk agar assay, and the *zmpA* mutant has approximately 20% of the activity of the parent, suggesting that these enzymes may work together to degrade casein, resulting in zones of clearance. The histopathologies observed in lungs of infected rats were similar for animals infected with either the *zmpA*, *zmpB*, or *zmpA zmpB* double mutant, again suggesting that the activities of these enzymes are not additive, since knocking out either gene results in a similar reduction in virulence.

Previously, we have reported that mutations in *cepI*, *cepR*, *cciI*, or *cciR* result in decreased *zmpA* expression in K56-2 (40, 54). In this study, we determined that *zmpB* expression was also markedly reduced in *cepI*, *cepR*, *cciR*, and *cepR cciIR* mutants. The expression of the *zmpB*::*luxCDABE* fusion was not above the background fluorescence obtained with the pMS402 vector control in the *cepI* or *cepR* mutants, indicating that the *cepIR* quorum-sensing genes are required for expression of both *zmpB* and *zmpA*. These results correlate with the observed absence of protease activity in *cepI* and *cepR* mutants (32, 40). The *cciR* and *cepR cciR* mutants were also previously shown to have decreased protease activity compared to the parent strain, which correlates with the *zmpB* expression data obtained in this study. Interestingly, the *cciI* mutant expressed *zmpB*::*luxCDABE* at much higher levels than the parent or any of the other mutants. The increase in *zmpB* expression corresponds to the increased protease activity previously observed in the *cciI* mutant on skim milk agar (40). The zones of protease activity in the *cciI* mutant could be restored to parental levels by the addition of 2.5 nM *N*-hexanoyl-L-homoserine lactone, which is the major AHL synthesized by *cciI*. These data suggest that the protease activity observed in the *cciI* mutant is due to the increased expression of *zmpB* and that the observed decrease in *zmpA* expression has little impact on total protease activity. It is interesting that the presence of *zmpB* on a multicopy plasmid does not result in increased protease zone sizes similar to that observed in the *cciI* mutant but only restores protease activity to the parental level. This finding suggests that *zmpB* copy number is not sufficient for increased protease expression or activity but that other factors, such as the balance of AHL signals, have a significant influence on the amount of protease produced. Mutations in *cciI* and *cciR* result in different effects on *zmpB* expression and protease activity. These mutants also have different swarming phenotypes, suggesting that other genes must also be differentially expressed in *cciI* and *cciR* mutants (40).

The ZmpB precursor is processed into mature ZmpB and a propeptide with a predicted molecular mass of 23.1 kDa and a pI of 8.8. The propeptide mass and pI correspond to a peptide identified in a proteome comparison between *B. cenocepacia* H111 and an H111 *cepI* mutant (46). The QS8 ID spot was identified as an intracellular thermolysin metallopeptidase and presumed to be a degradation product of a precursor metalloprotease. The N-terminal sequence of QS8 was determined to be DTKVSN, which corresponds to the predicted N terminus of the ZmpB propeptide after removal of the pre-signal peptide (46). Peptide QS8 was found to be approximately 20-fold up-regulated in the H111 parent compared to the *cepI* mutant, which is consistent with our data indicating that *zmpB* is positively regulated by the *cepIR* system.

In summary, we have shown that *B. cenocepacia* has two zinc metalloproteases that contribute to virulence and have the potential to interfere with host defense mechanisms. Further studies are under way in our laboratory to determine the in vivo activities of these proteases and their mechanisms of regulation.

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