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Distribution and Expression of the ZmpA Metalloprotease in the *Burkholderia cepacia* Complex

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The distribution of the metalloprotease gene *zmpA* was determined among strains of the *Burkholderia cepacia* complex (Bcc). The *zmpA* gene was present in *B. cepacia*, *B. cenocepacia*, *B. stabilis*, *B. ambifaria* and *B. pyrrocinia* but absent from *B. multivorans*, *B. vietnamiensis*, *B. dolosa*, and *B. anthina*. The presence of *zmpA* generally correlated with extracellular proteolytic activity with the exception of five strains, which had *zmpA* but had no detectable proteolytic activity when skim milk agar was used as a substrate (*zmpA* protease deficient). Western immunoblot experiments with anti-ZmpA antibodies suggest that the *zmpA* protease-deficient strains do not secrete or accumulate detectable ZmpA. Transcriptional *zmpA*::*lacZ* fusions were introduced in selected strains of the Bcc. *zmpA*::*lacZ* was expressed in all strains, but expression was generally lower in the *zmpA* protease-deficient strains than in the *zmpA* protease-proficient strains. Quantitative reverse transcriptase real-time PCR demonstrated that *zmpA* protease-deficient strains did express *zmpA* mRNA, although at various levels. *ZmpA* has previously been shown to be positively regulated by the CepIR quorum-sensing system. Addition of exogenous AHLs did not restore extracellular protease production to any of the *zmpA* protease-deficient strains; however, introduction of *cepR* in *trans* complemented protease activity in two of five strains. Extracellular proteolytic activity was restored by the presence of *zmpA* in *trans* in two of the five strains. These studies suggest that although some strains of the Bcc contain the *zmpA* gene, multiple factors may influence its expression.

The *Burkholderia cepacia* complex (Bcc) is a group of opportunistic pathogens that can cause severe respiratory infections in individuals with cystic fibrosis (CF) (25, 28, 41). These organisms are resistant to most available antibiotics, and some strains are highly transmissible. Chronic colonization with the Bcc is associated with a more rapid deterioration of lung function than either colonization with *Pseudomonas aeruginosa* alone or no colonization (10, 41). The Bcc presently consists of nine species: *B. cepacia* (formerly genomovar I) (45), *B. multivorans* (formerly genomovar II) (45), *B. cenocepacia* (formerly genomovar III) (44), *B. stabilis* (formerly genomovar IV) (46), *B. vietnamiensis* (formerly genomovar V) (15), *B. dolosa* (formerly genomovar VI) (6, 47), *B. ambifaria* (formerly genomovar VII) (7), *B. anthina* (formerly genomovar VIII) (43), and *B. pyrrocinia* (formerly genomovar IX) (43). Recent studies have revealed heterogeneity in the *recA* gene sequence among different isolates of *B. cenocepacia*, which led to the distinction of four *recA* lineages, referred to as IIIA, IIIB, IIIC, and IIID (26, 44).

Cell-cell signaling is used by certain bacteria to monitor their own population density and modulate gene expression accordingly once a critical cell density has been reached (12). Two sets of cell-cell signaling genes are present in *B. cenocepacia*. The *cepIR* genes are widely distributed in the Bcc (16, 23), whereas the *cciIR* genes are only found in *B. cenocepacia* strains containing the *cci* genomic island (1). The *N*-acyl homoserine lactones (AHLs) produced by CepI and CciI have been identified as *N*-octanoyl-homoserine lactone (OHL) and *N*-hexanoyl-homoserine lactone (HHL) (23, 30). Both the *cepIR* and *cciIR* genes influence protease production (30, 40).

A potential virulence trait that may contribute to the severity of Bcc infections is the ability to secrete extracellular proteases. A total of 69 to 88% of clinical isolates from the Bcc have been reported to produce extracellular proteases (13, 14, 31, 32). Variability in virulence has been shown between and within species of the Bcc (4, 5, 18, 22, 29, 37); thus, it is possible that the ability to produce extracellular proteases may account for some of these differences.

ZmpA (formerly PSCP) is a zinc metalloprotease originally described in *B. cenocepacia* Pc715j (31). We have shown that ZmpA is an important virulence factor in some strains, since a K56-2 *zmpA* mutant was less able to persist in a chronic lung infection model and caused less pathological damage to the lung than the parent strain, K56-2 (9). Interestingly, a *zmpA* mutant in strain Pc715j was similar in virulence to the parent strain, suggesting that other proteases in this strain may compensate for the *zmpA* defect (9). ZmpA is able to degrade several biologically important substrates, including neutrophil α-1 proteinase inhibitor, α_2-macroglobulin, gamma interferon, type IV collagen, and fibronectin, which may account for its ability to cause tissue damage as well as modulate the host immune system (19). The objectives of the present study were to determine the distribution and expression of the *zmpA* gene within different species of the Bcc.

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MATERIALS AND METHODS

Strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are described in Table 1. *Escherichia coli* DH5α and strains of the Bcc were routinely grown at 37°C in Luria-Bertani broth (LB) (Invitrogen,

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description (location)	Source or reference
Strains		
E. coli		
DH5α	F$^-$ ϕ80d*lacZ*ΔM15(*lacZYA-argF*) *recA1 endA gyrA96 thi-1 hsdR17 supE44 relA1 deoR U169*	Invitrogen
TOP10	F$^-$ *mrcA* Δ(*mrr-hsdRMS-mrcBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (Sm^r) *endA1 nupG*	Invitrogen
B. cepacia		
ATCC 25416^T	Environmental isolate, onion (United States)	27
ATCC 17759	Environmental isolate, soil (Trinidad)	27
CEP 509	CF isolate (Australia)	27
LMG 17997	Urinary tract infection (Sweden)	27
B. multivorans		
C5393	CF isolate (Canada)	27
LMG 13010	CF isolate (Belgium)	27
C1576	CF isolate, epidemic (United Kingdom)	27
CF-A1-1	CF isolate, epidemic (United Kingdom)	27
JTC	Chronic granulomatous disease CGD (United States)	27
C1962	Clinical isolate (United Kingdom)	27
ATCC 17616	Soil (United States)	27
249-2	Laboratory strain (United States)	27
B. cenocepacia		
J2315	CF isolate, epidemic (United Kingdom)	27
K56-2	CF respiratory isolate, *recA* lineage IIIc, BCESM$^+$; *cblA*$^+$ (Canada)[a]	27
K56-I2	*cepI*::Tp derivative of K56-2; Tp^r	20
Pc715j	CF isolate (Canada)	31
C5424	CF isolate, epidemic (Canada)	27
C6433	CF isolate, epidemic (Canada)	27
C1394	CF isolate, epidemic (United Kingdom)	27
PC184	CF isolate, epidemic (United States)	27
CEP 511	CF isolate, epidemic (Australia)	27
J415	CF isolate (United Kingdom)	27
ATCC 17765	Urinary tract infection (United Kingdom)	27
B. stabilis		
LMG 14294	CF isolate (Belgium)	27
LMG 14086	Respirator (United Kingdom)	27
C7322	CF isolate (Canada)	27
LMG 18888	Clinical isolate (Belgium)	27
B. vietnamiensis		
PC259	CF isolate (United States)	27
FC441	CGD (Canada)	27
LMG 16232	CF isolate (Sweden)	27
LMG 10929^T	Rice rhizosphere (Vietnam)	27
B. dolosa		
L06	CF isolate	6
LMG 18943	CF isolate (United States)	6
LMG 19468^T	CF isolate (United States)	J. LiPuma
B. ambifaria		
LMG 17828	Corn roots, biocontrol (United States)	7
LMG 19182^T	Pea rhizosphere, biocontrol (United States)	7
LMG 19467	CF isolate (Australia)	7
B. anthina		
LMG 20980^T	Soil (United States)	8
LMG 20982	Hospital environment (United Kingdom)	J. R. W. Govan
LMG 20983	CF isolate (United Kingdom)	8
LMG 16670	Rhizosphere (United Kingdom)	8
B. pyrrocinia		
LMG 21822	Soil (United States)	8
LMG 14191^T	Soil (Japan)	8

Continued on following page

TABLE 1—*Continued*

Strain or plasmid	Description (location)	Source or reference
Agrobacterium tumefaciens A136	Ti plasmidless host	C. Fuqua
Plasmids		
pCR2.1-TOPO	TOPO-TA cloning vector, pUC ori, p*lac*, *lacZ*α; Ap^r Km^r	Invitrogen
pUCP28T	Broad-host-range vector, IncP OriT pRO1600 ori; Tp^r	38
p34E-tp	Source of trimethoprim cassette; Tp^r	11
pSG200	pUCP28T with 2.6-kb PstI fragment from Pc715j containing *zmpA* inserted in the PstI site of the multiple cloning site; Tp^r	This study
pSG208	pUCP28T containing *zmpA* on a 2.6-kb PstI fragment with a SmaI *lacZ*::Gm^r fragment from pZ1918G inserted in the StuI site of *zmpA*; Gm^r Tp^r	40
pZ1918	Source of *lacZ* reporter; Ap^r Gm^r	38
pBS7	pCR2.1TOPO with 1.3-kb fragment containing *ndh*	This study
pTOPOZMPA	pCR2.1TOPO::*zmpA*; Ap^r Km^r	9
pSLR100	pUCP28T containing a 1.6-kb KpnI-SphI fragment with *cepR*; Tp^r	21
pCF218	IncP *traR*; Tc^r	50
pMV26	*traI-luxCDABE*; Km^r	40

[a] BCESM, *B. cepacia* epidemic strain marker.

Burlington, Ontario, Canada) or on 1.5% LB agar plates. For protease assays, dialyzed brain heart infusion agar containing 1.5% skim milk (D-BHI milk) (39) was used. For AHL extraction and quantification, strains from the Bcc were grown in tryptic soy broth (BD Diagnostic Systems, Franklin Lakes, N.J.) at 30°C for 12 h. The following concentrations of antibiotics were used when necessary: 100 μg of ampicillin, 50 μg of kanamycin, and 800 μg of trimethoprim per ml for *E. coli* and 100 μg, 400 μg, or 800 μg of trimethoprim per ml for the Bcc.

DNA manipulations. Molecular biology techniques were performed as generally described by Sambrook et al. (35). Genomic DNA was isolated according to the method of Walsh et al. (48). Recombinant plasmids were electroporated into *E. coli* DH5α or Bcc cells using a Gene Pulser (Bio-Rad, Richmond, CA) according to the manufacturer's instructions or transformed into *E. coli* DH5α cells made competent by treatment with $CaCl_2$ (24). For Southern hybridization, approximately 200 ng of DNA was digested with PstI, separated by agarose gel electrophoresis, and transferred to GeneScreen II membrane (Perkin-Elmer). The blot was hybridized with a 250-bp PCR product amplified with primers 1-2RRP3 and 1-2FFP2 as described below and labeled with ^{32}P with the Rediprime II Random Prime Labeling System (Amersham Biosciences).

PCR conditions. The *zmpA* gene was amplified using oligonucleotide primers 1-2RRP3 (CCAACCTGAACTATTCGG) and 1-2FFP2 (TGCGGATCGTGGCGC) or PNPF (ATCACCTTCTAGCATCC) and PNPR (CCTTCTCGTCATTCACC). PCRs were performed in the AB Applied Biosystems GeneAmp PCR System 2400 as follows: initial denaturation at 96°C for 4 min, denaturation at 96°C for 30 s, primer annealing at 56°C for the amplification of *zmpA* for 30 s, and extension at 72°C for 4.5 min for 30 cycles, with a final 10-min extension at 72°C. PCRs were routinely run on a 0.8 to 1.0% agarose gel, and bands were visualized by being stained with ethidium bromide. PCR products were purified from the agarose gel with the QIAquick gel extraction kit (Qiagen, Mississauga, Ontario, Canada) according to the manufacturer's instructions. Cloning of PCR products was performed using the TOPO-TA cloning kit into vector pCR2.1-TOPO, according to the manufacturer's directions (Invitrogen).

Real-time quantitative reverse transcriptase PCR (RT-PCR). Total RNA was isolated using RiboPure bacteria (Ambion, Austin, TX) from approximately 10 A_{600} U of culture incubated for 18 h at 37°C with shaking at 250 rpm. Residual DNA was removed by treatment with RNase-free DNase I using 8 U of TURBO DNase, followed by DNase Inactivation reagent (Ambion); the concentration was determined by measuring absorbance at 260 nm. To confirm that residual DNA was removed, control PCRs were performed using Platinum*Taq* DNA polymerase (Invitrogen Canada, Inc.) and the Fndh and Rndh primers. Oligonucleotide primer sequences were designed with Primer Express software, version 2 (Applied Biosystems, Foster City, CA), based on the unpublished genome sequence of *B. cenocepacia* J2315 (http://www.sanger.ac.uk/Projects/B_cenocepacia/). Primers EBS-E62 (5′-CGCGGCAGACATCGACTAC-3′) and RBS-E62 (5′-AGATGCCGTTGCGGTTGT-3′) were used to amplify *zmpA*. Primers Fndh (5′-GCGATCGGGCTGTACAAGTT-3′) and Rndh (5′-AGTGGCTCAGCGACTGGAA-3′) were used to amplify the NADH dehydrogenase gene (*ndh*) (BCAM0166) as a control gene, since *ndh* was expressed at a similar level in all strains examined.

Purified *zmpA* and *ndh* RNAs were used to construct standard curves. pTOPOZMPA (9) was digested with SpeI and purified with a Qiagen gel extraction kit (Qiagen, Mississauga, ON). The linear plasmid was used as a template to synthesize RNA using the T7 Ribomax Express Large Scale RNA Production system (Promega, Madison, WI). RNA was purified with a RNeasy Mini Kit (Qiagen), according to the manufacturer's recommendations. Primers (5′-ATGGACAACACCACGCCCAC-3′) and (5′-TCAGTGCAGCTTGATCGACG-3′) were used to amplify a 1.3-kb fragment containing *ndh* which was cloned into pCR2.1Topo (Invitrogen) downstream of the T7 promoter, generating pBS7. In vitro transcription of pBS7 was performed as above. The purified *zmpA* and *ndh* RNAs, in concentrations ranging from 20 pg to 0.2 fg, were used to construct standard curves for relative quantification with the TaqMan instrument and SYBR green RT-PCR reagents (Applied Biosystems). RT-PCR was performed with 25 μl (1× SYBR Green PCR Master Mix, 0.25-U/ml MultiScript reverse transcriptase, 0.4-U/ml RNase inhibitor, and 50 nM forward and reverse primers) using the following PCR cycles: 1 cycle at 48°C for 30 min; 1 cycle at 95°C for 10 min; and 45 cycles, each at 95°C for 15 s and 60°C for 1 min. Signal intensity was measured at the end of each elongation phase. A standard curve was generated from the cycle threshold of standard dilution series by the TaqMan 7500 system SDS software (version 1.3). All quantitative RT-PCRs (qRT-PCRs) were performed twice in triplicate.

β-Galactosidase assays. Expression of *zmpA* was quantified using a liquid β-galactosidase assay (34). Overnight starter cultures were subcultured 1/200 in 50 ml of medium and grown at 37°C for 20 h with shaking at 200 rpm. All assays were conducted in triplicate and repeated at least twice. Values shown represent the percentage of activity of the mean Miller units obtained with the K56-2 control cultures.

Protease assay on D-BHI skim milk plates. Overnight cultures were subcultured 1/50 in 50 ml of culture. Cells were grown until mid-log to late log phase (optical density at 600 nm [OD_{600}] = 0.5 to 1.0) and normalized to an OD_{600} of 0.3; 3 μl was spotted in triplicate onto D-BHI milk plates (39). Plates were incubated at 37°C for up to 48 h and examined for zones of clearing around the colonies at 24 and 48 h by determining the length of the radius (from the outside of the colony to the edge of the zone). Each protease assay was repeated at least twice in triplicate.

AHL extraction and quantification. *Agrobacterium tumefaciens* A136 (pCF218) (pMV26) was grown at 30°C in LB or on LB solidified with 1.5% agar. Medium was supplemented with 25 μg of kanamycin per ml and 4.5 μg of tetracycline per ml or 3 μg tetracycline per ml in liquid broth as required. AHLs were extracted from the following strains: *B. cepacia* CEP 509; *B. cenocepacia* K56-2, ATCC 17765, CEP 511, J415, C5424, and J2315; *B. stabilis* LMG 14086 and LMG 14294; and *B. ambifaria* LMG 19182^T as described previously (20). AHLs were quantitated with the *luxCDABE* reporter *Agrobacterium tumefaciens* A136-

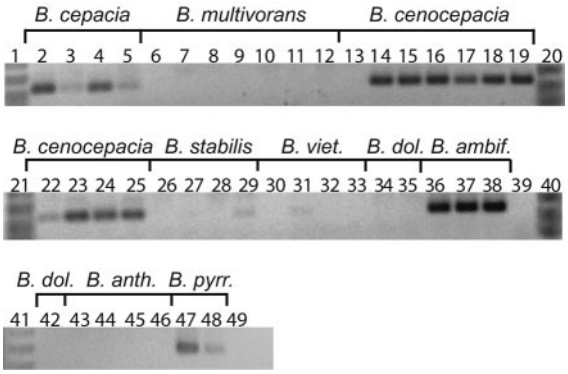

FIG. 1. Detection of *zmpA* in the *B. cepacia* complex by PCR. Lanes: 1, 1-kb Plus DNA ladder (Invitrogen); 2, ATCC 25416^T; 3, ATCC 17759; 4, CEP 509; 5, LMG 17997; 6, C5393; 7, LMG 13010; 8, C1576; 9, CF-A1-1; 10, JTC; 11, C1962; 12, ATCC 17616; 13, 249-2; 14, J2315; 15, Pc715j; 16, K56-2; 17, C5424; 18, C6433; 19, C1394; 20, 1-kb Plus DNA ladder (Invitrogen); 21, 1-kb Plus DNA ladder (Invitrogen); 22, PC184; 23, CEP 511; 24, J415; 25, ATCC 17765; 26, LMG 14294; 27, C7322; 28, LMG 14086; 29, LMG 18888; 30, PC259; 31, LMG 16232; 32, FC441; 33, LMG 10929^T; 34, L06; 35, LMG 18943; 36, LMG 17828; 37, LMG 19182^T; 38, LMG 19467; 39, H_2O control; 40, 1-kb Plus DNA ladder (Invitrogen); 41, 1-kb Plus DNA ladder (Invitrogen); 42, LMG 19468^T; 43, LMG 20980^T; 44, LMG 20983; 45, LMG 16670; 46, LMG 20982; 47, LMG 21822; 48, LMG 14191^T; and 49, H_2O control. *B. viet*., *B. vietnamiensis*; *B. dol*., *B. dolosa*; *B. ambif.*, *B. ambifaria*; *B. anth.*, *B. anthina*; and *B. pyrr.*, *B. pyrrocinia*.

(pCF218)(pMV26) as previously described (40). Briefly, AHL extracts were diluted 10 fold, and dilutions from 1/1,000 to 1/1,000,000 were assayed using an overnight culture of *A. tumefaciens* A136(pCF218)(pMV26). Ten microliters of extract, 10 μl of *A. tumefaciens* A136(pCF218)(pMV26), and 80 μl of LB were mixed in 96-well black plates with a clear flat bottom (Costar; Corning, Inc.). Luminescence was measured using a Wallac Trilux Luminescence counter (Perkin-Elmer Life Sciences) after 8 h of incubation at 30°C. Synthetic OHL (Sigma-Aldrich, Oakville, Ontario, Canada) was used to prepare a standard curve for comparison.

Cross-feeding assays. To determine if the addition of exogenous AHLs would restore protease production to *zmpA* protease-deficient strains, cross-feeding and AHL addback assays were performed. The following strains were tested: *B. cepacia* CEP 509 and *B. cenocepacia* J415, CEP 511, C5424, and J2315. *B. cenocepacia* K56-I2, a *cepI*::tp mutant shown to be protease negative but able to have protease production restored with the addition of exogenous AHLs (20), was used as a control. *B. cenocepacia* K56-2 was streaked perpendicularly to the test strains on D-BHI milk plates (39). The ability of *zmpA* protease-deficient strains to restore protease production to *B. cenocepacia* K56-I2 by the production of sufficient AHLs was also determined. Plates were incubated for up to 48 h and observed for a zone of clearing around the junction, which would indicate restoration of extracellular proteolytic activity by the AHLs produced by K56-2. Alternatively, 2 nmol of synthetic OHL and HHL (Fluka) were added to a filter disk (Becton Dickinson) and placed adjacent to the test strain, and the plates were observed for the restoration of protease production. Test strains were streaked perpendicularly to *B. cenocepacia* K56-I2 and observed for protease production at the junction.

Preparation of cell culture supernatants and extracts. *B. cepacia* CEP 509; *B. cenocepacia* Pc715j, K56-2, CEP 511, ATCC 17765, J2315, J415, and C5424; *B. stabilis* LMG 14086 and LMG 14294; and *B. ambifaria* LMG 19182^T were grown overnight, subcultured to a final OD_{600} of 0.05, and grown for 18 h with shaking at 200 rpm. Cultures were centrifuged at 10,000 × *g* for 1 h at 4°C. The resultant supernatants were precipitated by the slow addition of cold acetone-trichloracetic acid (final concentration, 10% [vol/vol]) for 4 h at 4°C with stirring, followed by placement at −20°C for 2 h and then at 4°C for 1 h. The precipitated culture supernatants were centrifuged at 15,000 × *g* for 1 h, and the pellets were washed three times with 100% acetone. After being air dried, the resultant pellets were resuspended in 30 mM Tris-HCl, pH 7.5. The amount of protein present was quantified by the method of Bradford with a protein assay kit (Bio-Rad Laboratories). Ten micrograms of each precipitated protein preparation was sepa-

TABLE 2. Distribution of protease gene and extracellular protease activity in the *Burkholderia cepacia* complex

Strain	*zmpA* detected by PCR	*zmpA* detected by Southern hybridization	Protease activity[a], avg radius ± SD (mm)
B. cepacia			
ATCC 25416^T	+	+	1.2 ± 0.3
ATCC 17759	+[b]		5.5 ± 0.0
CEP 509	+		0
LMG 17997	+[b]		5.0 ± 0.0
B. multivorans			
C5393	−	−	0
LMG 13010	−	−	0
C1576	−		0
CF-A1-1	−		0
JTC	−		0
C1962	−		0
ATCC 17616	−		0
249-2	−		0
B. cenocepacia			
J2315	+	+	0
K56-2	+	+	4.5 ± 0.5
Pc715j	+		6.0 ± 0.0
C5424	+		0
C6433	+		3.8 ± 0.3
C1394	+		0[c]
PC184	+[b]		0[c]
CEP 511	+		3.8 ± 0.7
J415	+		0[d]
ATCC 17765	+		5.0 ± 0.5
B. stabilis			
LMG 14294	+[b]	+	0
LMG 14086	+[b]	+	3.9 ± 0.1
C7322	+[b]		0[c]
LMG 18888	+[b]		3.3 ± 0.3
B. vietnamiensis			
PC259	−	−	0
FC441	−		0
LMG 16232	−	−	0
LMG 10929^T	−		0
B. dolosa			
L06	−		0
LMG 18943	−	−	0
LMG 19468^T	−	−	0
B. ambifaria			
LMG 17828	+	+	4.3 ± 0.3
LMG 19182^T	+		6.0 ± 0.0
LMG 19467	+	+	0.8 ± 0.3
B. anthina			
LMG 20980^T	−	−	0
LMG 20982	−	−	0
LMG 20983	−		0
LMG 16670	−		0
B. pyrrocinia			
LMG 21822	+	+	1.2 ± 0.3
LMG 14191^T	+		4.2 ± 0.3

[a] Protease activity at 24 h.
[b] Strains weakly positive by PCR.
[c] Protease negative at 24 h but had zones of clearing at 48 h: C1394, 7.5 ± 0.5; PC184, 6.0 ± 0; and C7322, 2.8 ± 0.3.
[d] Zones of clearing detected at 48 h were <1 mm.

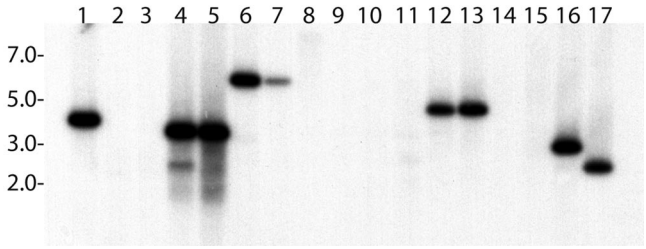

FIG. 2. Detection of *zmpA* in the *B. cepacia* complex by Southern hybridization. Southern blot of genomic DNA from representative strains digested with PstI, hybridized with a 250-bp fragment internal to *zmpA*, amplified by PCR, and labeled with ^{32}P. Lane 1, ATCC 25416; lane 2, C5393; lane 3, LMG 13010; lane 4, J2315; lane 5, K56-2; lane 6, LMG 14294; lane 7, LMG 14086; lane 8, PC259; lane 9, LMG 16232; lane 10, LMG 18943; lane 11, LMG 19468^T; lane 12, LMG 19467; lane 13, LMG 17828; lane 14, LMG 20980; lane 15, LMG 20982; lane 16, LMG 21822; lane 17, Pc715j.

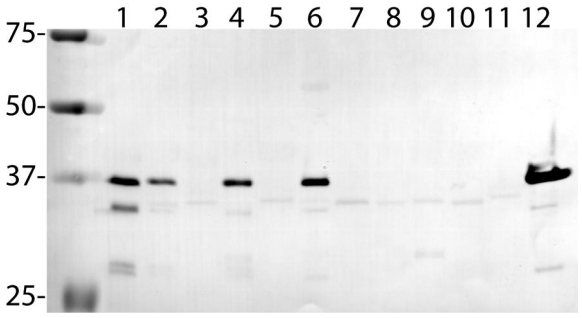

FIG. 3. Detection of ZmpA in culture supernatants. Western immunoblot of trichloroacetic acid-precipitated supernatants reacted with anti-ZmpA antibody. Lane 1, Pc715j; lane 2, K56-2; lane 3, Cep 511; lane 4, ATCC 17765; lane 5, LMG 14086; lane 6, LMG 19182; lane 7, Cep 509; lane 8, J2315; lane 9, J415; lane 10, C5424; lane 11, LMG 14294; lane 12, ZmpA.

rated by tricine–sodium dodecyl sulfate–14% polyacrylamide gel electrophoresis (36). For total cell extracts, cell pellets were resuspended in 30 mM Tris-HCl (pH 8.0), boiled for 20 min, and electrophoresed as above. Periplasmic extracts were prepared by resuspension of pellets from 100 ml of culture in 40 ml of 30 mM Tris-HCl (pH 8.0) containing 20% sucrose, followed by incubation at ambient temperatures for 20 min. The cells were centrifuged at 3,500 rpm for 20 min, resuspended in 2 ml of ice-cold 5 mM $MgSO_4$, incubated on ice for 20 min, and centrifuged as above; the supernatant enriched for periplasmic protein was collected and electrophoresed. Gels were transferred to polyvinylidene difluoride membranes (Millipore), essentially as described by Towbin et al. (42). After being blocked with 5% bovine serum albumin, the blots were reacted with rat anti-ZmpA antibody (19).

Nucleotide sequencing. Nucleotide sequencing was performed by either Macrogen, Inc. (Seoul, South Korea), or the University of Calgary Core DNA Services (Calgary, Alberta, Canada). Sequence analysis was performed with DNAMAN software (Lynnon Biosoft, Vaudreuil, Quebec, Canada). The *zmpA* sequences from strains K56-2, C5424, J415, and ATCC 17765 have been deposited in GenBank and assigned the following accession numbers, respectively: DQ069247, DQ06948, DQ069249, and DQ06950.

RESULTS

Distribution of the *zmpA gene* in the *Burkholderia cepacia* complex. Oligonucleotide primers 1-2RRP3 and 1-2FFP2 amplified a 228-bp PCR product from all strains of *B. cepacia*, *B. cenocepacia*, *B. ambifaria*, and *B. pyrrocinia* (Fig. 1) but not from strains of *B. multivorans*, *B. vietnamiensis*, *B. dolosa*, and *B. anthina*. A weak PCR product was consistently obtained for *B. cepacia* ATCC 17759 (lane 3) and LMG 17997 (lane 5), *B. cenocepacia* PC184 (lane 22), and *B. stabilis* LMG 14294 (lane 26), C7322 (lane 27), LMG 14086 (lane 28), and LMG 18888 (lane 29). To confirm that the faint 228-bp product from these strains was truly an amplicon of *zmpA*, these PCR products were cloned and sequenced. The deduced amino acid sequence of the products amplified from each of these strains was 95 to 100% identical to the portion of Pc715j ZmpA amplified with this set of primers, indicating that *zmpA* is present in these strains (data not shown). These results suggest that *zmpA* is present in *B. cepacia*, *B. cenocepacia*, *B. stabilis*, *B. ambifaria*, and *B. pyrrocinia* but absent from *B. multivorans*, *B. vietnamiensis*, *B. dolosa*, and *B. anthina* (Fig. 1 and Table 2).

To confirm that the species negative for *zmpA* by PCR analysis lacked the gene rather than contained a *zmpA* gene with nucleotide sequence variation in the primer binding site, Southern hybridization analysis with an internal *zmpA* probe was also performed with selected strains of each species (Fig. 2 and Table 2). Strains negative for *zmpA* by PCR did not hybridize with the *zmpA* probe; however, the probe clearly hybridized with two *B. stabilis* strains that were only weakly positive by PCR.

Protease activity in the *Burkholderia cepacia* complex. Corbett et al. (9) reported that a K56-2 *zmpA* isogenic mutant produced little or no zone of clearing on D-BHI skim milk plates, suggesting that in K56-2 ZmpA is the major extracellular protease capable of cleaving casein. To determine if the presence of *zmpA* correlated with casein-degrading activity, protease assays were performed on 40 strains of the Bcc. Most isolates from *B. cepacia*, *B. cenocepacia*, *B. stabilis*, *B. ambifaria*, and *B. pyrrocinia* were positive for extracellular proteolytic activity, but no isolates from *B. multivorans*, *B. vietnamiensis*, *B. dolosa*, and *B. anthina* had detectable extracellular proteolytic activity (Table 2), suggesting that protease activity generally correlated with the presence of *zmpA*.

B. cenocepacia C1394 and Pc184 were protease negative at 24 h but had zones comparable to K56-2 and Pc715j at 48 h (Table 2). *B. stabilis* C7322, although protease negative at 24 h, had zones comparable to those of the other *B. stabilis* strains by 48 h. These data suggest that induction of protease activity may be slower in these three strains but that they produce similar amounts of final activity.

Interestingly, *B. cepacia* CEP 509; *B. cenocepacia* J2315, C5424, and J415; and *B. stabilis* LMG 14294 had little or no detectable proteolytic activity despite the presence of the *zmpA* gene. J415 had a zone of clearing of <1 mm at 48 h. To determine if ZmpA was secreted by these strains, protein profiles of culture supernatants of these strains and selected *zmpA* protease-proficient strains (Pc715j, K56-2, CEP 511, ATCC 17765, LMG 14086, and LMG19182^T) were examined on Western blots reacted with anti-ZmpA. Anti-ZmpA reacted with an approximately 36-kDa protein in supernatants from *B. cenocepacia* Pc715j, K56-2, and ATCC 17765 and *B. ambifaria* LMG 19182^T. The *zmpA* protease-deficient strains and two of the *zmpA* protease-proficient strains (Cep 511 and LMG14086) did not react with the antibody to ZmpA (Fig. 3). These data suggest that *zmpA* may not be properly transcribed, translated, or secreted in the *zmpA* protease-deficient strains. To determine if there was an accumulation of ZmpA intracellularly in these strains,

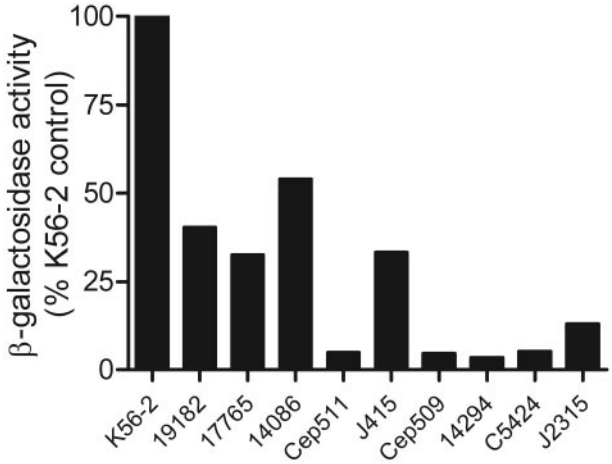

FIG. 4. Comparison of *zmpA*::*lacZ* expression in *zmpA* protease-proficient and *zmpA* protease-deficient strains. Expression of the *zmpA*::*lacZ* fusion contained on pSG208 was determined by measuring β-galactosidase assays. Assays were conducted in triplicate and repeated at least twice. Values shown are the mean percentages of K56-2 activity, which is represented as 100%, and are of a representative experiment. β-Galactosidase activity resulting from strains with the pUCP28T vector control was negligible and is not shown.

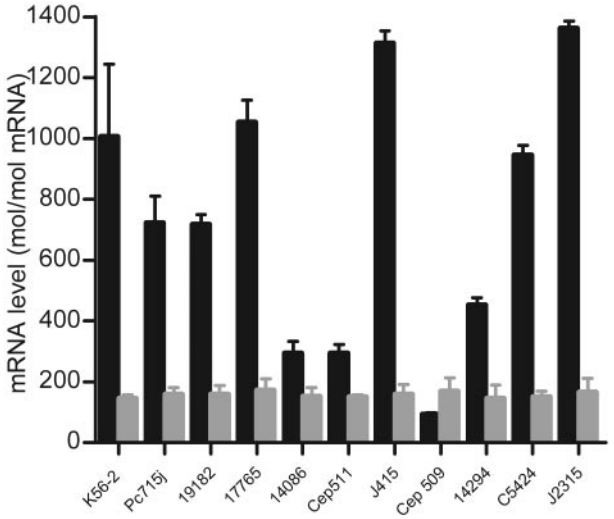

FIG. 5. Comparison of *zmpA* expression in *zmpA* protease-proficient and *zmpA* protease-deficient strains by quantitative real-time RT-PCR. Expression of *zmpA* and *ndh* were determined by absolute quantification. Concentrations of the mRNA level of each strain are expressed as moles of target gene mRNA per mole compared to the concentration of the standard curve. The bars represent the means ± standard deviation of triplicate assays. Black bars, *zmpA*; gray bars, *ndh*.

whole-cell lysates and periplasmic extracts were also examined by Western blotting. No ZmpA was detected in either fraction from either the *zmpA* protease-proficient or the *zmpA* protease-deficient strains (data not shown).

Expression of *zmpA* in the *Burkholderia cepacia* complex. To determine why strains J2315, C5424, J415, LMG 14294, and CEP 509, which contain *zmpA*, lack extracellular protease activity, the ability of these strains to express *zmpA* was compared to selected *zmpA* protease-proficient strains. Plasmids pSG208, which contains a *zmpA*::*lacZ* transcriptional reporter fusion (40), and pUCP28T (vector control) were introduced in the *zmpA* protease-proficient strains K56-2, LMG 19182^T, ATCC 17765, LMG 14086, and CEP 511 and the *zmpA* protease-deficient strains J2315, C5424, J415, LMG 14294, and CEP 509. Plasmid pSG208 was unstable in Pc715j. The expression of the *zmpA*::*lacZ* transcriptional fusion was determined by β-galactosidase assays (Fig. 4). β-Galactosidase activity was generally higher in the protease-proficient strains than in the protease-deficient strains, with the exception of strains Cep511 and J415.

Expression of *zmpA* was also measured by quantitative real-time reverse transcriptase PCR. All of the strains expressed *zmpA* mRNA, but the level of expression did not correlate with activity on D-BHI milk plates (Fig. 5). Together, these experiments suggest that all of the strains that contain *zmpA* have the ability to express this gene, although the expression levels vary considerably among strains.

Complementation of *zmpA* protease-negative strains. One possible explanation for the absence of detectable extracellular proteolytic activity in the *zmpA* protease-deficient strains is that *zmpA* contains a mutation that affects enzyme activity in these strains. Recently, the genomic sequence of *B. cenocepacia* J2315 was completed by the Wellcome Trust Sanger Institute (www.sanger.ac.uk/Projects/B_cepacia/). The J2315 *zmpA* gene is 99.5% identical to the *B. cenocepacia* Pc715j *zmpA* gene (9). The only difference between the amino acid sequences is a substitution of a serine at residue 489 in Pc715j for the threonine in J2315. The ability of Pc715j *zmpA* to restore protease activity in strains LMG 14294, C5424, J415, J2315, and CEP 509 was determined by introducing pSG200, a high-copy-number plasmid, with *zmpA*. Extracellular protease activity was restored in J415 and C5424 when *zmpA* was provided in *trans*, but not in LMG 14294, J2315, or CEP 509 (Fig. 6).

The ability of Pc715j *zmpA* to restore protease activity in J415 and C5424 suggests that *zmpA* in these strains may contain a mutation that alters enzyme activity. The J415 and C5424 *zmpA* genes were amplified by PCR using primers PNPF and PNPR, and their nucleotide sequences were determined. C5424 ZmpA was 99.82% identical to Pc715j ZmpA and contained a threonine at residue 489. J415 ZmpA was 96.81% identical to Pc715j ZmpA and contained 18 amino acid changes, including a threonine at residue 489. To determine if this serine-threonine substitution correlated with the lack of extracellular proteolytic activity, we sequenced *zmpA* from two *zmpA* protease-proficient strains, K56-2 and ATCC 17765 (data not shown). Both strains also harbored a threonine at position 489, indicating that the absence of extracellular proteolytic activity in *B. cenocepacia* C5424, J2315, and J415 could not be explained by threonine in this position. There were no other consistent sequence differences that could account for the lack of protease activity in these strains.

Effect of *cepI* and *cepR* on *zmpA* expression. We have recently demonstrated that the *cepIR* cell-cell signaling system positively regulates the expression of *zmpA* at the transcriptional level (40). To determine if the lack of protease activity in the *zmpA* protease-deficient strains might be due to differences in *cepI* or *cepR*, the effects of exogenous AHL and the ability of *cepR* in *trans* to restore protease activity were determined. AHLs were extracted from the *zmpA* protease-proficient and

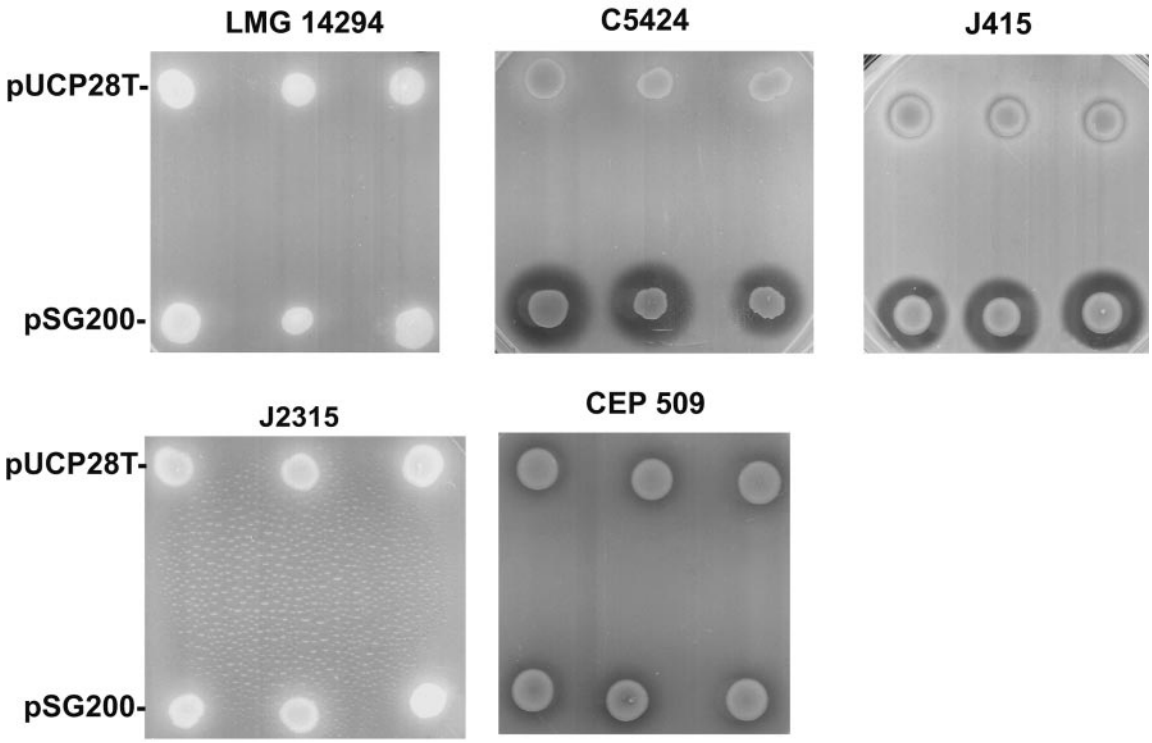

FIG. 6. Complementation of *zmpA* protease-deficient strains with Pc715j *zmpA* in *trans*. The assay shown is representative of two experiments performed in triplicate. Only strains J415 and C5424 had extracellular proteolytic activity when harboring pSG200. Strain CEP 509 produces a dark melanin-like pigment, which is visible around the colonies, but no zone of clearing was detectable.

zmpA protease-deficient strains and tested for activity as previously described (40). AHL activity was detected in all the strains tested (data not shown). Cross-feeding assays were also used to determine if the lack of protease activity was related to AHL production. The *zmpA* protease-deficient strains were streaked perpendicularly to *B. cenocepacia* K56-2 (which produces both OHL and HHL) or supplemented with synthetic OHL or HHL to determine if the availability of exogenous AHLs would restore extracellular protease activity (21). Neither cross-streaking with *B. cenocepacia* K56-2 nor the addition of synthetic OHL or HHL was able to restore protease production in the *zmpA* protease-deficient strains (data not shown), indicating that the lack of protease activity was not due to concentrations of AHLs insufficient to activate *zmpA* expression. We also tested for the ability of the *zmpA* protease-deficient strains to restore extracellular proteolytic activity in a *cepI* mutant, K56-I2. All *zmpA* protease-deficient strains were able to restore protease activity to K56-I2 in the cross-streaking assay, confirming that these strains produce sufficient functional AHLs to activate transcription of the *zmpA* gene by CepR. The ability of *cepR*, introduced on a high-copy-number plasmid (pSLR100) to restore protease activity to the *zmpA* protease-deficient strains, was also determined (Table 3). Introduction of pSLR100 increased protease activity in J2315 and C5424 compared to the same strains without the plasmid or with the vector control.

The predicted promoter sequences of J2315 and Pc715j *zmpA* were compared and found to be identical in both the −35 (TTGTAA) and −10 (TTCTAGCAT) regions (www.softberry.com). The predicted *cep* box sequence upstream of *zmpA* was also identical in these strains, suggesting that although an increase in *cepR* copy number results in protease activity in J2315, the CepR binding site is not altered in J2315. The J3215 *cepR* sequence is identical to that of K56-2, indicating that the difference in activity is not due to a *cepR* mutation.

DISCUSSION

The distribution of *zmpA* correlated with Bcc species. *zmpA* was detected in *B. cepacia*, *B. cenocepacia*, *B. stabilis*, *B. ambifaria*, and *B. pyrrocinia* but absent from *B. multivorans*, *B. vietnamiensis*, *B. dolosa*, and *B. anthina*. Strains possessing *zmpA* had proteolytic activity on D-BHI milk agar, whereas strains from which the gene could not be amplified had no protease activity, suggesting that ZmpA is responsible for most

TABLE 3. Complementation of protease activity by pSLR100 (*cepR*)[a]

Strain	Activity with:		
	No plasmid	pUCP28T	pSLR100
K56-2	6.5 ± 0	6.7 ± 0.3	6.8 ± 0.3
Cep 509	0.5 ± 0	0.5 ± 0	0.5 ± 0
J2315	0.3 ± 0	0 ± 0	1.3 ± 0
J415	0.3 ± 0	0.3 ± 0	0.3 ± 0
C5424	0 ± 0	0 ± 0	0.9 ± 0
LMG14294	0.2 ± 0	0 ± 0	0.5 ± 0

[a] Zones of clearing on D-BHI milk agar at 48 h. Values are average radius ± standard deviation in millimeters.

of the protease activity in this assay. Five strains (*B. cepacia* CEP 509; *B. cenocepacia* C5424, J2315, and J415; and *B. stabilis* LMG 14294) had little or no detectable proteolytic activity or detectable ZmpA, despite the presence of the *zmpA* gene. No intracellular accumulation of either ZmpA or its precursor form, preproZmpA, was detectable, which would be indicative of a defect in secretion; however, it is possible that ZmpA would be degraded if it were not secreted properly.

A functional *zmpA* gene present in *trans* was able to restore protease production in strains C5424 and J415, suggesting that the *zmpA* genes in these strains contained mutations that affected their activity or that the increase in copy number of *zmpA* due to the plasmid produced levels of expression sufficient to result in activity. J415 expressed both the *zmpA*::*lacZ* fusion and *zmpA* mRNA detectable by qRT-PCR at fairly high levels, suggesting that J415 *zmpA* may have a mutation that decreases activity, but this strain is capable of producing extracellular protease activity when carrying a functional copy of *zmpA*. J415 ZmpA has 18 amino acids that differ from Pc715j. It is possible that one or more of these alterations affect enzyme activity.

Interestingly, strain C5424 also produced detectable protease activity when *cepR* was introduced on a plasmid. Multiple copies of *cepR* might directly increase expression of either the *zmpA* gene or another gene involved in *zmpA* expression or secretion. Introduction of *cepR* also restored protease activity to J2315, which suggests that the *zmpA* gene is functional in this strain but that a regulatory factor is deficient, since *zmpA* on a high-copy-number plasmid did not result in protease activity. Exogenous OHL was not able to restore protease activity to either C5424 or J2315. These results suggest that the increased protease activity induced by *cepR* is not due to an increase in OHL production as a result of enhanced *cepI* expression but may be due to another as-yet-unidentified gene regulated by CepR.

Strains LMG 14294 and CEP509 were not proteolytically active with either a functional copy of *zmpA* or *cepR* in *trans*, suggesting that these strains have other defects in protease expression. These strains had lower expression of both *zmpA* mRNA detectable by qRT-PCR and the *zmpA*::*lacZ* fusion than most of the *zmpA* protease-proficient strains.

In *Pseudomonas aeruginosa*, several global regulators have been shown to contribute to the regulation of secondary metabolites and virulence determinants at the posttranscriptional level including Vfr (2, 3, 49), DksA (17), and RsmA (33). Vrf, a homologue of *Escherichia coli* cyclic AMP receptor protein, positively regulates elastase and pyocyanin production via activation of the transcriptional regulator LasR (2). Posttranscriptional control by DskA is required for full translation of the *lasB* elastase gene and the rhamnosyltransferase-encoding *rhlAB* (17). Interestingly, RsmA is a global posttranscriptional negative regulator of extracellular enzymes and *N*-AHL production (33). RsmA controls the temporal expression of the *lasI* gene, and *lasI* is induced earlier and at a higher level during the exponential growth phase in a *rsmA* mutant. It is possible that LMG 14294 and Cep509 lack a regulator of protease production. Studies are in progress to identify potential global regulators involved in *zmpA* expression in *B. cenocepacia*.

Western immunoblot experiments with anti-ZmpA antibodies suggest that the *zmpA* protease-deficient strains do not secrete detectable ZmpA or that the possibly inactive products are degraded. The antibody did not react with two of the *zmpA* protease-proficient strains, which may indicate that ZmpA is not secreted from LMG 14086 and CEP 511 or is produced in very small amounts. It is possible that the polyclonal antibodies to ZmpA do not recognize ZmpA from all species in the complex, although the antibodies did react with ZmpA in several strains of *B. cenocepacia* and *B. ambifara* LMG 19182. It is also possible that the extracellular proteolytic activity observed on D-BHI milk agar in these strains was due to other extracellular proteases.

We have determined that, although ZmpA may be present in many strains and species of the Bcc, there are likely multiple factors influencing its expression including the *cepIR* quorum-sensing system. ZmpA has been shown to be an important virulence factor in some strains of *B. cenocepacia* (9). The majority of *B. cenocepacia* strains are capable of producing protease, but expression varies and may be influenced by a number of factors. It is possible that J2315 and C5424 produce protease activity in vivo, due to increased expression of either *cepR* or *zmpA*. Strains C1394 and PC 184 have no protease activity at 24 h yet have significant activity on plates by 48 h, suggesting that expression of *zmpA* is delayed in these strains. Strains of *B. multivorans*, which have been shown to be generally less virulent than *B. cenocepacia* in both a chronic lung infection model and an alfalfa infection model (4), lack the *zmpA* gene and protease activity in the conditions examined. It is possible that the absence of *zmpA* accounts for some of the reduced virulence of *B. multivorans*.

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