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Molecular Characterization of a Secreted Enzyme with Phospholipase B Activity from *Moraxella bovis*

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A candidate for a vaccine against infectious bovine keratoconjunctivitis (IBK) has been cloned and characterized from *Moraxella bovis*. The *plb* gene encodes a protein of 616 amino acids (molecular mass of ~65.8 kDa) that expresses phospholipase B activity. Amino acid sequence analysis revealed that PLB is a new member of the GDSL (Gly-Asp-Ser-Leu) family of lipolytic enzymes.

Moraxella bovis is the etiologic agent of infectious bovine keratoconjunctivitis (IBK), the most common ocular disease that occurs in cattle (2, 13, 15). IBK is highly contagious and, if left untreated, can result in corneal ulceration and temporary or permanent blindness. In vitro studies have suggested that *M. bovis* produces a number of virulence determinants, including type IV pili (3, 23), a secreted hemolysin (28), proteases, fibrinolysins, and phospholipases (9). To date, only type IV pili and the hemolysin have been substantively linked to the virulence of *M. bovis* (4, 10, 16, 17, 20, 26). Here we describe the molecular characterization of an *M. bovis* gene encoding a phospholipase B activity and discuss the role this enzyme may play in the virulence of this veterinary pathogen.

The Australian *M. bovis* strains used in this study have been defined according to their pilus serotype (25): S276R (serotype A), 3WO7 (serotype B), Dalton 2d (serotype C), R593L (serotype D), Tat849 (serotype E), 218R (serotype F), and Fleur462 (serotype G). Other *M. bovis* strains used, Q220 and Epp63, were isolated in the United Kingdom and the United States, respectively. All of these *M. bovis* strains were positive for lipase activity when tested on Tween 80 medium (30), as indicated by a zone of opalescence around areas of growth.

Cloning and sequence analysis of the *M. bovis plb* gene. Genomic DNA from *M. bovis* strain Dalton 2d (serotype C) was purified with cetyltrimethylammonium bromide-NaCl and phenol chloroform extractions followed by isopropanol precipitation. The genomic DNA was partially digested with *Sau*3A under conditions that maximized the amount of DNA in the size range of 1 to 2 kb. Fragments <200 bp were removed by passing the digested DNA through a Microspin S-400 HR column (Pharmacia). The 1- to 2-kb fragments were ligated with pBR322 (5), previously digested with *Bam*HI, and electroporated into *Escherichia coli* strain MC1061 (31). The partial genome library was screened on Tween 80 medium, and 28 out of 24,000 clones were found to be positive for lipase expression.

All positive clones contained a 5.4-kb fragment in common. The size of the insert DNA in one positive clone, pMB1, was reduced from 5.4 kb by restriction endonuclease digestion to 2.2 kb (pMB4, positive for lipase expression) and 2.0 kb (pMB5, negative for lipase expression). It is interesting to note that neither MC1061/pMB1 nor MC1061/pMB4 was found to be positive for protease or hemolytic activity when plated onto agar containing skim milk or erythrocytes.

Recombinant pMB4 DNA was isolated by using the Wizard Plus SV Minipreps DNA purification kit (Promega), and the nucleotide sequence of the 2.2-kb insert was determined (GenBank accession no. AY032849). An open reading frame of 1,851 bp with the potential to encode a 616-amino-acid protein with a predicted molecular mass of 65.8 kDa was identified together with a potential Shine-Dalgarno site preceded by putative promoter sequences. The sequence following the open reading frame has the potential to encode a transcriptional terminator with a ΔG value of -15 kJ mol^{-1}. Based on subsequent protein and biochemical analyses described below, this gene was designated *plb*.

Identification of the *plb* gene product. Secreted protein samples were prepared from *M. bovis* Dalton 2d and *E. coli* strains MC1061/pMB1, MC1061/pMB4, and MC1061/pMB5 by ammonium sulfate precipitation of cell supernatant fluids from overnight cultures, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12.5% polyacrylamide) (19), and visualized by staining with Coomassie blue. A dominant band running at approximately 66 kDa was identified in the extracts from MC1061/pMB1 and MC1061/pMB4, but not in those from MC1061/pMB5 (Fig. 1A, lanes a, b, and c, respectively). Due to the large number of proteins present in the *M.* bovis culture supernatant (contributed by the membrane blebs that slough off *M. bovis* during normal growth), Western blot analysis with antiserum raised in rabbits immunized with the recombinant lipase was required to confirm the presence of the lipase in the concentrated extract of *M. bovis* Dalton 2d-secreted proteins (Fig. 1B, lane d).

The dominant 66-kDa band identified in the MC1061/pMB1 preparation (Fig. 1A, lane a) was electrotransferred to polyvinylidene difluoride membrane, excised, and subjected to auto-

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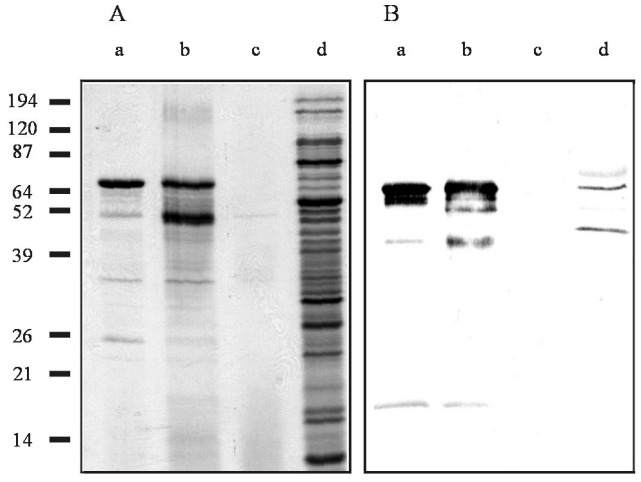

FIG. 1. Identification of native (*M. bovis*) and recombinant (*E. coli*) lipase. (A) SDS-PAGE gel stained with Coomassie brilliant blue. (B) Western blot of the gel in panel A probed with antisera raised in rabbits against heat-inactivated recombinant lipase. Lanes: a, MC1061/pMB1; b, MC1061/pMB4; c, MC1061/pMB5; d, *M. bovis* Dalton 2d. Molecular mass markers are indicated in kilodaltons to the left.

mated (Edman degradation) amino-terminal sequence analysis (7). The resultant data confirmed the identity of the 66-kDa protein as the product of *plb* and furthermore identified the presence of a signal peptide cleavage site between residues 25 and 26 of the predicted translation product, designated PLB. This putative signal peptide has similar characteristics to classical signal peptides described by Pugsley (27), suggesting that PLB may be secreted across the cytoplasmic membrane via an *s*-dependent pathway.

Amino acid sequence comparisons. Comparison of the PLB sequence with the GenBank sequence databank by using the BLAST program (1) revealed a number of similar proteins. PLB had its closest identity (28% identity and 42% similarity) to a lipase or esterase from the plant pathogen *Xylella fastidiosa* (accession no. D82761). Three other proteins, including a putative autotransporter protein (PapA; accession no. CAC14200) and an esterase (EstA; accession no. G83006) from *Pseudomonas aeruginosa* and an outer membrane esterase from *Salmonella enterica* serovar Typhimurium (AAC38796), displayed 23, 23, and 31% identity and 35, 35, and 43% similarity, respectively, to PLB. *M. bovis* PLB was also found to be related to a group of prokaryotic and eukaryotic proteins by virtue of the presence of a highly conserved amino acid sequence motif, Gly-Asp-Ser-Leu (GDSL) (29), located in the N-terminal regions of each of these proteins (Fig. 2). While variable in size and appearing to have different functions (29, 32), the proteins aligned share five discrete blocks of relatively high sequence similarity that occur in the same relative location in each protein. The serine residue within the GDSL motif has been shown to be part of the catalytic triad of the *Aeromonas hydrophila* glycerophospholipid-cholesterol acyltrans-

	Organism	Accession No.	Function	Block 1	
				*********	
a.	*Moraxella bovis*	AY032849	lipase/phospholipase B	VIIFGDSLSDTG	(48)
b.	*Aeromonas hydrophila*	P10480	lipase/acyltransferase	IVMFGDSLSDTG	(37)
c.	*Salmonella typhimurium*	AF047014	esterase	LTVIGDSLSDTG	(31)
d.	*Escherichia coli*	P29679	thioesterase/protease	LLILGDSLS-AG	(27)
e.	*Xylella fastidiosa*	D82761	lipase/esterase	TIFFGDSLSDSG	(36)
f.	*Vibrio cholerae*	U50074	lecithinase	VIAFGDSLSDTG	(36)
g.	*Pseudomonas putida*	P40604	ORF in trpE-trpG region	MIVFGDSLSDAG	(48)
h.	*Pseudomonas aeruginosa*	G83006	lipase/esterase	LVVFGDSLSDAG	(63)
i.	*Vibrio parahaemolyticus*	Q99289	haemolysin	VVALGDSLSDTG	(36)
j.	Xenorhabdus *luminescens*	P40601	lipase	LYVFGDSLSDGG	(34)
k.	*Arabidopsis thaliana*	P40604	proline-rich protein	VFFFGDSVFDTG	(62)
l.	*Brassica napus*	P40603	proline-rich protein	VFFFGDSIFDTG	(68)
m.	Acidiphilum sp.	AB026254	thermostable acid esterase	LYVFGDSLSDDG	(?)

	Block 2		Block 3		Block 4		Block 5
	* * * ***		* ** ****		**** * * **		*** ***
a.	PTGTNYAVGGAR	(36)	YAIWIGSNDLI	(31)	AGATTILVPNVPD	(106)	FADDIHPS
b.	LTIANEAEGGPT	(37)	VILWVGANDYL	(26)	NGAKEILLFNLPD	(128)	FWDQVHPT
c.	NGGSNYAAGGAT	(32)	YIHWVGGNDLA	(30)	AGAGLVVVPNVPD	(156)	FADHLHPG
d.	ISGDTSQQGLAR	(12)	VLVELGGNDGL	(36)	YGRRYNEAFSAIY	(26)	QDDGIHPN
e.	ASPNGNGQTGNN	(42)	YTLWGGGNDLL	(29)	AGARYIVVATIPD	(89)	FADDIHPT
f.	VPLYNWAVGGAA	(34)	FTLEFGLNDFM	(22)	AGAKNLVLMTLPD	(101)	FWEVTHPT
g.	PDGNNWAVGGYT	(45)	YYLTGGGNDFL	(26)	GGARYIMVWLLPD	(94)	FNDLVHPT
h.	ADGNNWAVGGYR	(45)	YYITGGGNDFL	(26)	AGARYIVVWLLPD	(92)	FNDSVHPT
i.	LPLYNWAVGGAA	(34)	FTLEFGLNDFM	(22)	AGAKNFMLMTLPD	(101)	FWNVTHPT
j.	KGGTNYAAGGAT	(31)	YVHWIGGNDVD	(29)	AGAGLVIVPTVPD	(155)	FADDFHPT
k.	LTGVSFASGGAG	(55)	AIVVGGSNDLI	(35)	YGARRIGVIGTPP	(101)	FWDGVHPT
l.	LTGVSFASGGAG	(45)	AIVVAGSNDLI	(35)	YGARRIGVIGTPP	(101)	FWDGLHPS
m.	????????????	(?)	VTLWGGANNYF	(37)	LGARMLIVPNLPD	(89)	FWDNVHPT

FIG. 2. Conserved blocks of amino acids in the GDSL family (members b to m) of lipolytic enzymes. New family member *M. bovis* PLB (member a) has been compared with proteins of those organisms shown to express a protein belonging to the GDSL family. The accession number for each protein sequence is indicated, as is the function of each enzyme. Asterisks indicate residues that are conserved in six or more of the proteins. Numbers in parentheses indicate the number of amino acid residues between the conserved regions. ORF, open reading frame. The figure was adapted from data provided in the study of Upton and Buckley (29).

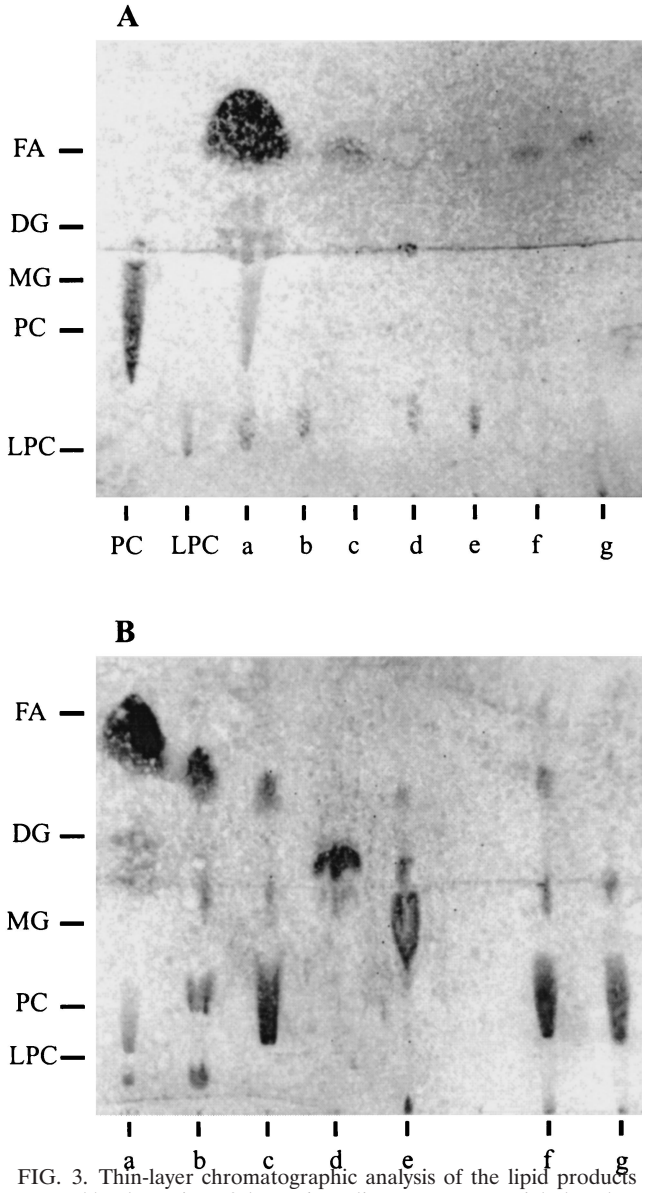

FIG. 3. Thin-layer chromatographic analysis of the lipid products generated by the action of the *M. bovis* lipase or commercial phospholipases on two different substrates: phosphatidylcholine (PC) (A) and lysophosphatidylcholine (LPC) (B). Lanes: a, reference standards; b, phospholipase A_2; c, phospholipase B; d, phospholipase C; e, phospholipase D; f, MC1061/pMB4; g, *M. bovis* Dalton 2d. Included as controls in panel A are unprocessed phosphatidylcholine and lysophosphatidylcholine. The other reference standards used were monoacyl glycerol (MG), diacyl glycerol (DG), and free fatty acid (FA).

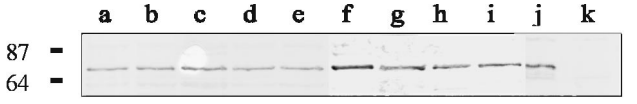

FIG. 4. Distribution of PLB among *M. bovis* strains. Secreted proteins present in cell supernatants prepared from overnight cultures of *M. bovis* strains were precipitated with ammonium sulfate at a final concentration of 60%. Proteins were visualized by Western blot analysis with recombinant PLB antisera. Lanes: a, S276R; b, 3WO7; c, Dalton 2d; d, R593L; e, Tat849; f, 218R; g, Fleur462; h, Q220; i, Epp63; j, MC1061/pMB4; k, MC1061/pMB5. Molecular mass markers are indicated in kilodaltons to the left.

ferase (14) and may therefore be essential for enzymatic activity, and possibly the virulence, of members of this family. We propose that the PLB from *M. bovis* is a member of the GDSL family and as such may play a role in the pathogenesis of the IBK infection. The precise nature of this role will be investigated by constructing a *plb* mutant and testing the pathogenicity of the mutant in the bovine IBK model system (20).

It has been suggested that one member of the GDSL family, the EstA esterase of *P. aeruginosa,* may also be a member of the autotransporter protein family (32). Autotransporters are secreted across the cytoplasmic membrane via the *s*-system and then secreted across the outer membrane by a unique process that does not require any energy or additional accessory factors (12, 22). The C-terminal region of PLB was found to display homology to members of the autotransporter family. Taken together with the observation that PLB probably has a classical signal peptide, we propose that PLB is a bona fide member of the autotransporter family.

Characterization of PLB enzymatic activity. To determine the enzymatic specificity of PLB, thin-layer chromatography was used to analyze the end products of phosphatidylcholine and lysophosphatidylcholine hydrolysis (8). Ammonium sulfate-precipitated cell supernatant fluids from *E. coli* MC1061/pMB4 and *M. bovis* Dalton 2d hydrolyzed both substrates to produce free fatty acids and glycerophosphorylcholine in a manner similar to that observed for the commercial phospholipase B (Fig. 3). Based on these data, we concluded that the activity displayed by PLB was characteristic of a phospholipase B.

Expression of PLB by *M. bovis*. To investigate the frequency of expression of PLB among isolates of *M. bovis*, a Western blot using antisera raised in rabbits immunized with the recombinant lipase was performed with protein extracts from nine representative strains. Protein samples were made from overnight cultures, separated by SDS-PAGE (12.5% polyacrylamide), transferred to nitrocellulose, and incubated with recombinant lipase antisera. A protein band equivalent in size (~66 kDa) to the lipase produced by *E. coli* MC1061/pMB4 (Fig. 4, lane j) was detected in each of the *M. bovis* strains (Fig. 4, lanes a to i), thus establishing the global nature of this enzyme among the species.

Previous studies have shown that the type IV pili produced by *M. bovis* play a fundamental role in bacterial colonization of the bovine eye and mediate the essential first step in the pathogenic process that leads to disease (20). Following adhesion, secondary virulence factors are presumed to cause the clinical damage observed as pitting on the corneal surface (18). Enzymatic activity of the *M. bovis* phospholipase B on membrane phospholipids could result in cell lysis and lead to such clinical symptoms. This damage could be directly caused by the presence of lysophosphatidylcholine, one of the intermediate products of PLB activity on phospholipids (6). Phospholipases are recognized as major virulence determinants in a number of bacterial species, including *Listeria* (11) and *Corynebacterium pseudotuberculosis* (24).

Until now, development of a pilus-based vaccine against IBK has been hampered by the need to construct multivalent vac-

cines, since the pili of specific serotypes are not cross protective against disease caused by heterologous challenge (21). The failure of such vaccines supports the need for the identification of immunogens that are antigenically conserved across *M. bovis*. The identification of PLB as one such conserved immunogen will likely prove important in the future development of an efficacious vaccine against IBK.

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