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Biochemical and Molecular Analysis of Phospholipase C and Phospholipase D Activity in Mycobacteria

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Resurgence of mycobacterial infections in the United States has led to an intense effort to identify potential virulence determinants in the genus *Mycobacterium*, particularly ones that would be associated with the more virulent species (e.g., *Mycobacterium tuberculosis*). Thin-layer chromatography (TLC) using radiolabeled phosphatidylcholine and sphingomyelin as substrates indicated that cell extracts of *M. tuberculosis* contain both phospholipase C (PLC) and phospholipase D (PLD) activities. In contrast, only PLD activity was detected in cell extracts of *M. smegmatis*. Neither activity was detected in cell-free culture supernatants from these organisms. We and others recently identified two open reading frames in *M. tuberculosis* with the potential to encode proteins which are highly homologous to the nonhemolytic (PlcN) and hemolytic (PlcH) phospholipase C enzymes of *Pseudomonas aeruginosa*. In contrast to the *plc* genes in *P. aeruginosa*, which are considerably distal to each other (min 34 and 64 on the chromosome), the mycobacterial genes, designated *mpcA* and *mpcB*, are tandemly arranged in the same relative orientation and separated by only 191 bp. Both the *mpcA* and the *mpcB* genes were individually cloned in *M. smegmatis*, and PLC activity was expressed from each gene in this organism. Hybridization experiments using the *mpcA* and the *mpcB* genes as probes under conditions of moderate stringency identified sequences homologous to these genes in *M. bovis*, *M. bovis* BCG, and *M. marinum* but not in several other *Mycobacterium* species, including *M. smegmatis*, *M. avium*, and *M. intracellulare*. TLC analysis using radiolabeled substrates indicated that *M. bovis* and *M. marinum* cell extracts contain PLC and PLD activities, but only PLD activity was detected in *M. bovis* BCG cell extracts. Sphingomyelinase activity was also detected in whole-cell extracts of *M. tuberculosis*, *M. marinum*, *M. bovis*, and *M. bovis* BCG, but this activity was not detected in extracts of *M. smegmatis*. Sphingomyelinase activity was detected in cell extracts from *M. smegmatis* harboring either recombinant *mpcA* or *mpcB*. These data indicate that PLC and sphingomyelinase activities are associated with the most virulent mycobacterial species, while PLD activity was detected in both virulent and saprophytic strains.

Phospholipases have been demonstrated to be important virulence factors in an increasing number of bacteria, including *Clostridium perfringens* (12, 34), *Corynebacterium pseudotuberculosis* (10, 19), the intracellular pathogen *Listeria monocytogenes* (1, 27), and the extracellular opportunistic pathogen *Pseudomonas aeruginosa* (23, 25). Phospholipases are generally categorized by their substrate specificities (Fig. 1). The most important classes of phospholipases that have been thus far shown to play a significant role in bacterial pathogenesis are the phospholipase C (PLC) and phospholipase D (PLD) classes. For example, PLC enzymes cleave the phospholipid phosphatidylcholine to produce phosphorylcholine and diacylglycerol (DAG), while PLD enzymes cleave phosphatidylcholine to generate choline and phosphatidic acid (PA).

L. monocytogenes produces two PLC enzymes. The product of the *plcA* gene encodes a phosphatidylinositol-specific PLC, while the *plcB* gene product is a PLC with broad-range specificities (7, 35). Mutants with alterations in *plcA* are decreased in virulence (1). They are able to invade but not replicate within mouse peritoneal macrophages, suggesting that *plcA* mutants cannot efficiently escape from the host cell phagosome (1). While they are able to lyse single-membrane phagosomes, *plcB* mutants appear to be deficient in escape from the double-membrane vacuoles which result from cell-to-cell spread during infection with *L. monocytogenes* (35). More recently, it has been demonstrated that the two PLC activities act synergistically in contributing to pathogenesis (18, 33).

Since *P. aeruginosa* is primarily an extracellular pathogen, the role of PLC in pathogenesis is likely to be distinct from that in organisms such as *L. monocytogenes*. The hemolytic PLC of *P. aeruginosa*, PlcH, has been demonstrated to be a virulence factor for both plants and animals (23, 25). PlcH is highly toxic to mouse fibroblasts (NIH 3T3 cells), a human monocyte cell line, peripheral blood lymphocytes, and neutrophils, but it is not toxic to a human alveolar epithelial cell line even at high concentrations. It does, however, induce high levels of interleukin 8 expression in this kind of cell (2). Meyers and Berk (20) demonstrated that sublethal concentrations of PlcH induce de novo production of eicosinoids by macrophages and neutrophils. Thus, it is likely that the PlcH plays a role in the inflammation processes that are a hallmark of pulmonary infections caused by this organism, such as those seen in cystic fibrosis patients.

The production of signaling molecules, such as DAG, may be important in considering the ramifications of PLC activity in pathogenesis. DAG has been demonstrated to play a role in eukaryotic cell signaling events through the activation of protein kinase C. Moreover, downstream metabolites of DAG, such as arachidonic acid-derived eicosinoids, can contribute to the inflammatory process, as has been demonstrated for PlcH (20). PA, generated through PLD-mediated phospholipid hydrolysis, also has the potential to induce cell signaling molecules. One mechanism is by conversion of PA to DAG through the action of a eukaryotic PA phosphohydrolase (4). In addition, PA itself induces arachidonic acid release and thus can

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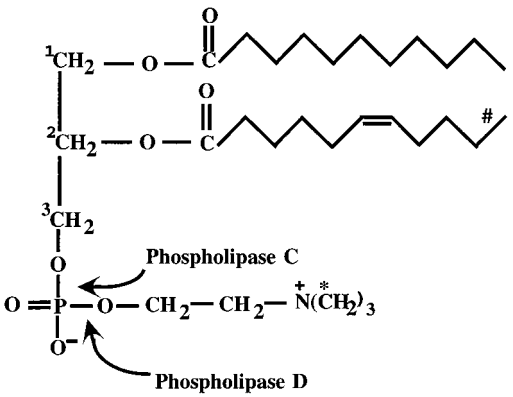

FIG. 1. Cleavage positions of phospholipases. The structure of phosphatidylcholine is shown, with cleavage positions of PLC and PLD (arrows) and radiolabeled carbon groups (choline labeled [asterisk] or chain labeled [pound sign]) used in assays to detect phospholipase activity indicated.

stimulate the production of inflammatory mediators. The production of ceramide through sphingomyelin hydrolysis also can induce signals which mimic the effect of tumor necrosis factor alpha (TNF-α) (26). Hydrolysis of phospholipids by bacterial phospholipases thus has the potential to profoundly alter cellular and immune responses.

Tuberculosis remains the most widespread infectious disease worldwide. Despite intense efforts to understand the biology of *Mycobacterium tuberculosis* infection, little is known about its virulence factors. A search of the GenBank database conducted by our laboratory revealed the presence of *M. tuberculosis* sequences homologous to the PLC genes of *P. aeruginosa*. In earlier studies, a different group presented data suggesting that the DNA sequences encoding this PLC were unique to *M. tuberculosis* and were not present in other *Mycobacterium* species (3, 24). The DNA fragments carrying these genes were thus initially identified as potential diagnostic tools for the specific identification of *M. tuberculosis* and only recently were reported to have sequences homologous to the *plc* genes of *P. aeruginosa*. Leão et al. (16) subsequently described the cloning and characterization of an entire gene homologous to the *plc* genes of *P. aeruginosa*, and they identified a partial open reading frame (ORF) that is also homologous to these genes. While they did not report expression of PLC activity from the recombinant gene in *Escherichia coli*, they did report that a fusion of the complete *plc* gene with the gene encoding glutathione *S*-transferase had hemolytic activity (16) in *E. coli*. For this study, we have designated the PLC genes *mpcA* and *mpcB* (for mycobacterial phospholipase C). Because of the potential for PLC enzymes to contribute to the pathogenesis of *M. tuberculosis* at the level of intracellular survival by inappropriate signaling or by direct cytotoxicity, we initiated a more detailed molecular characterization of *mpcA* and *mpcB* and a biochemical analysis of phospholipase activities in mycobacterial species.

MATERIALS AND METHODS

Bacterial species, plasmids, and DNA. Table 1 lists the bacterial species and plasmids used in this work. Gamma-irradiated *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, and wet *M. smegmatis* mc^{2}155 whole cells grown on glycerol-alanine salts (GAS) medium at 37°C for 2 weeks were obtained from John Belisle, Colorado State University, Ft. Collins, through the National Institute of Allergy and Infectious Diseases, as were gamma-irradiated *M. bovis* cells grown on sodium pyruvate-alanine salts (SPAS) medium. SPAS medium is identical to GAS medium except that sodium pyruvate is substituted for glycerol. Chromosomal DNAs from *M. tuberculosis* Erdman, H37Rv, and H37Ra and *M. smegmatis* mc^{2}155 were also obtained from John Belisle. Whole-cell extracts of *M. marinum* 1218R were a gift from Pamela Small, Rocky Mountain Laboratories, National Institutes of Health, Hamilton, Mont. *M. marinum* 1218R is virulent for fish and amphibians (32). Chromosomal DNA from *M. marinum* 1218R was also obtained from Pamela Small. *M. bovis* BCG cells and chromosomal DNA were obtained from Julie Inamine, Colorado State University. Chromosomal DNAs from *M. cookii*, *M. scrofulaceum*, *M. marinum* 11566, *M. bovis* BCG, *M. intracellulare*, *M. avium*, *M. xenopi*, *M. aurum*, and *M. fortuitum* were obtained from Vojo Deretic, University of Texas Health Science Center, San Antonio. The *E. coli-M. smegmatis* shuttle vector pMX1 was a gift of Clifton Barry, Rocky Mountain Laboratories. Antibiotics were used at the following concentrations: for *E. coli*, ampicillin at 50 μg/ml and hygromycin B at 100 μg/ml, and for *M. smegmatis*, hygromycin B at 100 μg/ml.

Enzymes and reagents for molecular biology procedures. Restriction enzymes were purchased from Gibco BRL, Gaithersburg, Md., and used according to the manufacturer's recommendations. *Taq* DNA polymerase was from Gibco BRL and was used in conjunction with a PCR Optimizer kit (InVitrogen, San Diego, Calif.) according to the manufacturer's instructions. Cloning and transformation of PCR products were performed according to the manufacturer's instructions by using an InVitrogen TA cloning kit. PCR products were initially cloned into the pCRII (InVitrogen) vector after isolation from low-melting-point agarose and purification using a Wizard Clean Up kit (Promega, Madison, Wis.). Dideoxy DNA sequencing was performed with a Taquence sequencing kit (United States Biochemicals, Cleveland, Ohio) according to the manufacturer's recommendations.

Sequence determination of *mpcB*. Cosmid clones containing the entire *mpc* region were identified from a library of *M. tuberculosis* H37Rv DNA in pYUB18, kindly provided by Julie Inamine. Cosmids were identified by hybridization to a labeled PCR product generated from *M. tuberculosis* H37Rv chromosomal DNA using primers deduced from the sequence of the 3-kb fragment containing the previously described and sequenced *mtp40* gene (GenBank accession no. M57952) (24). The primer coordinates were bp 1587 to 1607 and 1887 to 1907.

TABLE 1. Bacteria and plasmids used in this study

Bacterium or plasmid	Relevant characteristic(s)	Source or reference
Bacteria		
E. coli DH5α	*supE44* Δ*lacU169* (ϕlacZΔM15) *hsdR17 recA endA1 gyrA96 thi-1 relA1*	Gibco BRL
M. tuberculosis H37Rv	Virulent	John Belisle
M. tuberculosis H37Ra	Avirulent	John Belisle
M. smegmatis mc^{2}155	Avirulent	John Belisle
M. marinum 1218R	Virulent (fish)	Pamela Small
M. bovis	Virulent	John Belisle
M. bovis BCG-Pasteur	Avirulent	Julie Inamine
Plasmids		
pCRII	Km^r Ap^r; cloning vector for PCR products	InVitrogen
pPZ375(−)	Ap^r; cloning vector	Andrew Sage
pBS(+/−)	Ap^r; cloning vector	Stratagene
pMX1	Hyg^r; *E. coli-M. smegmatis* shuttle vector	Clifton Barry
pMX-mpcA	Hyg^r; contains *mpcA* structural gene	This study
pMX-mpcB	Hyg^r; contains *mpcB* structural gene	This study

Positive cosmids were screened for the presence of a hybridizing 3.0-kb *Bam*HI fragment, which was subsequently cloned into pBS(+) (Stratagene, La Jolla, Calif.). Our analysis of this 3.0-kb *Bam*HI fragment confirmed the presence of *mpcA* and also identified the 5′ end of a second homologous PLC gene, *mpcB* (data not shown). The adjacent downstream DNA, containing 3′ sequences of *mpcB*, was cloned from the same cosmid as an overlapping 3.7-kb *Pst*I fragment (data not shown). This fragment was cloned into the *Pst*I site in pBS(−) (Stratagene) in both orientations to determine the complete nucleotide sequence of *mpcB*. Sequences from a portion of this fragment had been previously deposited in the GenBank database under accession no. M57952 (24) and were used as a starting point to determine the DNA sequence of the remainder of the *mpcB* gene. Specific primers were synthesized on a Beckman Oligo 1000 synthesizer and were used to walk down the *mpcB* gene. The sequence was verified by determining the nucleotide sequences of both DNA strands. The deduced amino acid sequence of MpcB was determined by using MacDNAsis v3.0 software. Nucleotide and amino acid alignments were also determined by using MacDNAsis v3.0, while codon preference was determined by using MacVector 4.1.1 software. Putative promoter regions and ribosome binding sites were identified by homology to *E. coli* and mycobacterial promoters (9, 14, 30). Regions with potential secondary structure were identified by using Genetics Computer Group (Madison, Wis.) software.

PCR. Primers 5′CAGAGGCAGGGCAAATGA3′ and 5′CACGGCCATTG CTGATCAG3′, which anneal to bp 422 to 441 and 2151 to 2170, respectively, were used to amplify *mpcA* from *M. tuberculosis* H37Rv chromosomal DNA. After a denaturing step of 95°C for 1 min, the primers were annealed at 60°C for 1.5 min and then extended in the presence of all four deoxynucleotides at 72°C for 2 min. Amplification was continued for 30 cycles using a DNA thermal cycler 480 (Perkin-Elmer Cetus, Foster City, Calif.). A 1.75-kb fragment corresponding to the full-length *mpcA* gene was amplified by this protocol. Primers 5′CGTCA AGGAGGGCGCGTG3′ and 5′GCGTCGAGTGTCGAATCCCGT3′, which anneal to bp 2175 to 2192 and 4036 to 4056, respectively, were used to amplify *mpcB* from *M. tuberculosis* H37Rv chromosomal DNA. After denaturation at 95°C for 1 min, annealing was done at 59°C for 1.5 min, the primers were extended at 72°C for 2 min, and amplification was continued for 30 cycles. This protocol allowed amplification of a 1.88-kb fragment corresponding to the full-length *mpcB* gene.

Construction of pMX-mpcA and pMX-mpcB. The 1.75-kb *mpcA* and 1.88-kb *mpcB* PCR-generated gene fragments were cloned into pCRII, and the sequences of the inserts were determined to verify the orientation and integrity of the coding regions. The inserts were both excised from pCRII by *Xba*I-*Hin*dIII digestion, and the fragments were isolated from low-melting-temperature agarose. The *mpcA*- and *mpcB*-containing fragments were then cloned into pPZ375(−) which had been digested with *Xba*I and *Hin*dIII. This intermediate cloning step provided additional restriction enzyme sites for subsequent manipulations. The *mpcA* and *mpcB* gene fragments were then released from pPZ375(−) by digestion with *Kpn*I and *Hin*dIII and ligated into pMX1 digested with the same enzymes. The resulting recombinants, containing the 1.75-kb *mpcA* gene and the 1.88-kb *mpcB* gene, were called pMX-mpcA and pMX-mpcB, respectively.

Electroporation of *M. smegmatis*. *M. smegmatis* mc^{2}155 was transformed with pMX-mpcA and pMX-mpcB as previously described (11), with the following modifications. An overnight culture of *M. smegmatis* mc^{2}155 was subcultured 1:50 into 20 ml of brain heart infusion (Difco, Detroit, Mich.) broth supplemented with 0.1% Tween 80 and 0.5% (vol/vol) glycerol and grown to late log phase. The cells were harvested by centrifugation at 4,940 × *g* for 10 min at 4°C and then resuspended in 5 ml of ice-cold 10% glycerol. The cells were washed once more with 10% glycerol and then resuspended in a final volume of 1 ml of ice-cold 10% glycerol containing 0.1% Tween 80. The competent cells were then mixed with approximately 1 μg of pMX1, pMX-mpcA, or pMX-mpcB plasmid DNA resuspended in distilled water in a Bio-Rad (Hercules, Calif.) cuvette with a 0.2-cm gap width and electrotransformed with a Bio-Rad Gene Pulser and Pulse Controller set at 25 μF, 800 Ω, and 1.75 kV. Transformants were selected by growth at 37°C on brain heart infusion agar containing hygromycin B (100 μg/ml) (Sigma Chemical Co., St. Louis, Mo.). Colonies were usually evident after 2 to 3 days.

Southern hybridization. Chromosomal DNAs (approximately 1 μg each) from *M. tuberculosis* Erdman, H37Rv, and H37Ra, *M. bovis* BCG, *M. smegmatis* mc^{2}155, *M. fortuitum*, *M. marinum* 1218R and 11566, *M. cookii*, *M. scrofulaceum*, *M. aurum*, *M. avium*, *M. intracellulare*, *M. xenopi*, and *M. ulcerans* were digested with *Sal*I and separated on a 0.8% agarose gel. DNA from the gel was transferred to two Nytran (Schleicher & Schuell, Keene, N.H.) membranes simultaneously, as described by Sambrook et al. (29). The membranes were prehybridized in 33% formamide–10% dextran sulfate–1% sodium dodecyl sulfate (SDS)–1 M NaCl overnight at 42°C and then probed with full-length *mpcA* or *mpcB* gene probes generated by PCR as described above. The probes were labeled with [α-^{32}P]dCTP (Dupont NEN, Boston, Mass.) by using a RadPrime (Gibco BRL) labeling kit according to the manufacturer's instructions. Hybridization was allowed to proceed overnight at 42°C. The membranes were washed in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0)–0.1% SDS at 65°C for 1 h after an initial rinse in the same wash solution. Autoradiography was performed at −70°C.

Preparation of whole-cell extracts. Approximately 3 g (wet weight) of cells (or 1 g [wet weight] of gamma-irradiated cells) was resuspended in 5 ml of buffer A (50 mM Tris [pH 7.4], 50 mM NaCl, 5% glycerol) and disrupted by three 1-min pulses with 0.1-mm-diameter glass beads using a BeadBeater apparatus (BioSpec, Bartlesville, Okla.) according to the manufacturer's instructions. In some cases, cells from 5 ml of late-log-phase cultures were pelleted, resuspended in 1 ml of buffer A, and disintegrated with 0.1-mm-diameter zirconium beads by using a mini-BeadBeater apparatus. The beads were removed by centrifugation, and the extract was collected. The extracts were aliquoted and stored at −70°C until used in phospholipase assays.

Determination of phospholipase activity. Phospholipase activity was qualitatively determined by thin-layer chromatography (TLC) using an assay based on that of Camilli et al. (1), which detects lipid hydrolysis products. Briefly, 100 μl of whole-cell extract was mixed with 13.5 μg of phosphatidylcholine, 86.5 μl of buffer (250 mM Tris [pH 7.4], 0.25% deoxycholate), and 0.045 μCi of [^{14}C]phosphatidylcholine (Dupont NEN), with the fatty acid side chain moieties labeled, in a final reaction volume of 200 μl. The reaction mixtures were vortexed and then sonicated for 1 min in a bath sonicator to form micellular structures. The reaction mixtures were incubated overnight at 37°C. A 40-μl sample of each reaction mixture was applied to the origin of a Silica Gel G (Fisher Scientific Co., Pittsburgh, Pa.) TLC plate and allowed to ascend in a solvent system of petroleum ether-ethyl ether-acetic acid (50:50:1, vol/vol/vol) until the solvent front was within 2 cm of the top of the plate. The plates were air dried and subjected to autoradiography overnight at room temperature.

Sphingomyelinase activity was detected by using an assay based on that of Goldfine et al. (8). A substrate solution of 288 μg of sphingomyelin containing 0.25 μCi of [^{14}C]sphingomyelin per reaction mixture was dried under nitrogen gas and reconstituted in a 100-μl volume per reaction in buffer containing 100 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), 100 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 7.5), and 1.84% Triton X-100. The substrate solution was sonicated and briefly vortexed to form mixed micelles. A sample of whole-cell extract or purified *P. aeruginosa* PlcH enzyme, which has previously been shown to have sphingomyelinase activity (22), was added to the substrate solution so that the final reaction volume was 200 μl. Addition of salts or cations was not required for enzyme activity. The reaction mixtures were incubated for 2 h to overnight at 37°C and then mixed with 1.6 ml of chloroform-methanol (1:1, vol/vol) and 0.8 ml of 0.1 N HCl, and then the water-soluble hydrolysis products were extracted by vortexing. After centrifugation at 1,200 rpm for 7 min in a Beckman GS-15R centrifuge, 0.6 ml of the upper, aqueous phase from each reaction mixture was collected, and 5 ml of Ecoscint (National Diagnostics, Life Science Products, Inc., Denver, Colo.) scintillation cocktail was added. The radioactivity of the samples was determined with a Beckman LS 7000 scintillation counter. Reaction products containing at least four times the counts of a control without added enzyme in at least three separate determinations were considered positive for sphingomyelinase activity. Results were verified by TLC as described above, except that the radiolabeled substrate was sphingomyelin and products were separated in a solvent system of *n*-butanol–ethanol–acetic acid–water (8:2:3:1, vol/vol/vol/vol).

Nucleotide sequence accession number. Sequences described in this report have been deposited in GenBank under accession no. U49511.

RESULTS

Characterization of the *mpc* genetic region in *M. tuberculosis*. During the isolation of the *mpcA* gene, we identified a partial ORF containing 5′ sequences of *mpcB*. To characterize the *mpcB* gene further, we identified and cloned sequences encompassing the entire *mpc* genetic region. Figure 2A shows a partial restriction endonuclease map of the *mpc* genetic region in *M. tuberculosis*. The *mpcA* and *mpcB* genes are tandemly arranged but appear to comprise distinct cistrons, as promoter-like elements can be found upstream of the putative start site for each gene. Analysis of the regions upstream of the putative start site for *mpcA* revealed the presence of elements with homology to promoters from *E. coli*, mycobacteria, and streptomycetes at bp 368 to 373, 387 to 392, and 399 to 304 (Fig. 2B and C). Sequences with homology to ribosome binding sites were also identified at bp 423 to 426. Folding predictions suggested that a region of secondary structure could form just upstream of the putative *mpcA* start site (Fig. 2A). The 1,563-bp *mpcA* ORF would encode a polypeptide of 521 amino acid residues, with a molecular weight of 56,103 and a pI of 5.99. The deduced amino acid sequence of MpcA indicates that it is homologous to the PLC enzymes of *P. aeruginosa*. MpcA has 36 and 38% overall amino acid identity with PlcH and PlcN, respectively, with the N-terminal regions showing

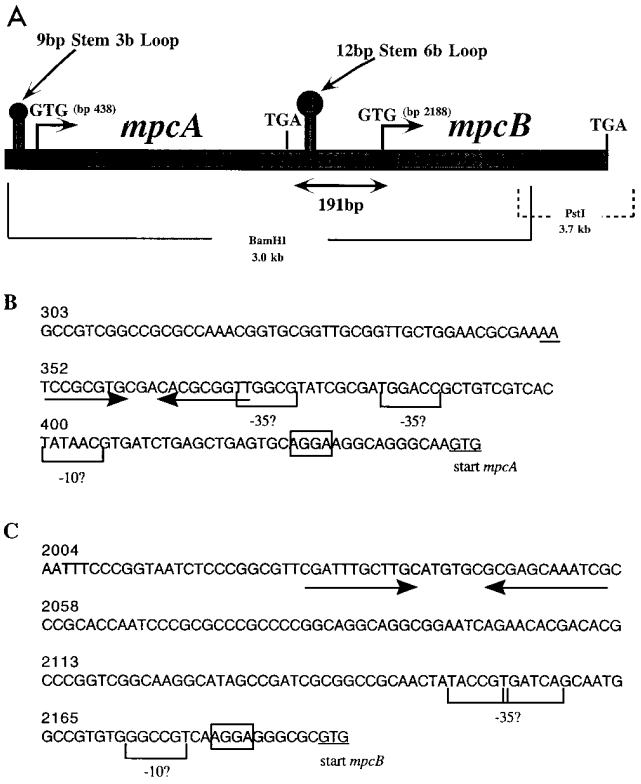

FIG. 2. (A) Organization of the *mpc* locus and the restriction endonuclease fragments used to localize *mpcA* and *mpcB*. The location and direction of transcription of *mpcA* and *mpcB* (arrows) are indicated. (B and C) Nucleotide sequences of the putative promoter regions for *mpcA* and *mpcB*, respectively. Sequences which can potentially form secondary structures (arrows), regions with homology to −35 and −10 promoters from *E. coli* and mycobacteria (brackets), possible ribosome binding sites (boxes), and the GTG start codons for *mpcA* (at bp 438) and *mpcB* (at bp 2188) (underlined) are indicated. Note that *mpcA* stops with a TGA codon at bp 1997 (not shown). Abbreviations: B, *Bam*HI; E, *Eco*RI; N, *Nde*I; P, *Pst*I; and S, *Sal*I.

greater homology. A putative signal sequence from MpcA is also similar to those of PlcH and PlcN, which are unusual in their long length and the presence of charged residues and phenylalanine.

Earlier analysis suggested the presence of an ORF downstream of *mpcA*, but the location and complete sequence of the *mpcB* gene were not determined in that study (16). We localized the 3′ portion of the *mpcB* gene by Southern hybridization studies to a 3.7-kb *Pst*I fragment which overlaps with the 3.0-kb *Bam*HI fragment containing all of *mpcA* and part of *mpcB* (Fig. 2A; data not shown). Contrary to an earlier report (16), we suggest that the *mpcB* gene starts at bp 2188 (Fig. 2B). This is because of the potential for formation of a secondary structure just downstream of the stop codon for *mpcA*. Should such a structure form, it would occlude the start site for *mpcB* predicted by Leão et al. (16). Codon preference analysis also suggests that the *mpcB* ORF starts downstream of this structure (data not shown). In addition, we identified elements with homology to known promoter sequences and ribosome binding sites at bp 2148 to 2158, 2170 to 2175, and 2178 to 2181 (Fig. 2B). Thus, we predict that *mpcB* starts with the GTG codon at bp 2188. The 3′ portion of the *mpcB* gene was identified, and its nucleotide sequence was determined. The 1,563-bp *mpcB* ORF would encode a polypeptide of 521 amino acids, with a molecular weight of 56,137 and a pI of 5.24. The nucleotide sequence of *mpcB* is 75% identical to that of *mpcA*, while the deduced amino acid sequence indicates that MpcB is 72% identical to MpcA at the protein level. MpcB is also homologous to the PLC enzymes of *P. aeruginosa*, PlcH and PlcN (29.6 and 38.5% overall identity, respectively). MpcB also contains a putative signal sequence with homology to those of MpcA, PlcH, and PlcN. Figure 3 shows an alignment of the deduced amino acid sequences of the PLC enzymes from *M. tuberculosis* and *P. aeruginosa*.

Distribution of *mpcA* and *mpcB* among mycobacteria. To verify that *mpcA* sequences are unique to *M. tuberculosis*, we examined chromosomal DNAs from a number of *Mycobacterium* species for the presence of *mpcA* by hybridization. Southern hybridization analysis identified two additional species of mycobacteria that contain sequences which hybridize to both *mpcA* and *mpcB* (Fig. 4). In contrast to earlier reports suggesting that sequences within *mpcA* are unique to *M. tuberculosis* (3, 24), *M. bovis* BCG and *M. marinum* were found to have sequences which hybridize to both the *mpcA* and the *mpcB* gene probes. DNA from *M. smegmatis* mc^{2}155, *M. fortuitum*, *M. cookii*, *M. scrofulaceum*, *M. aurum*, *M. avium*, *M. intracellulare*, and *M. xenopi* did not have any hybridizing sequences.

The autoradiogram shown in Fig. 4 indicates that *mpcA* and *mpcB* cross-hybridize under the hybridization conditions used. A 1.8-kb *Sal*I fragment, corresponding to *mpcB*, as well as a 2.7-kb fragment corresponding to *mpcA* in chromosomal DNAs from *M. tuberculosis* Erdman, H37Rv, and H37Ra hybridizes to both probes. An additional fragment of 3.0 kb also hybridizes with *M. tuberculosis* H37Ra DNA. A single 3.8-kb band from *M. bovis* BCG hybridizes to both *mpcA* and *mpcB* probes. *M. bovis* chromosomal DNA also contains a 3.8-kb fragment which hybridizes to both probes; in addition, it contains a fragment of 6.0 kb which hybridizes to an *mpcA* probe (data not shown). DNAs from *M. marinum* 11566 and 1218R contain a 1.7-kb fragment which hybridizes to both *mpcA* and *mpcB*. *M. marinum* 11566 also has a 2.3-kb fragment which hybridizes to both probes. This band is missing in *M. marinum* 1218R DNA and could reflect a restriction fragment length polymorphism. An additional 3.7-kb fragment is present in *M. marinum* 11566 DNA but not in DNA from *M. marinum* 1218R. Larger fragments (3.5, 5.5, and 7.5 kb) are present in DNAs from both strains and could reflect partial digestion due to methylation.

Expression of PLC and PLD activity in mycobacteria. We examined whole-cell extracts from various mycobacteria to determine if phospholipase activity was detectable. Figure 5 shows the results of TLC to analyze the lipid hydrolysis products of phospholipase activity. Whole-cell extracts from gamma-irradiated *M. tuberculosis* H37Rv cells were found to produce PA and DAG upon incubation with phosphatidylcholine ^{14}C labeled at the lipid moiety. Identical results were also obtained with whole-cell extract from *M. tuberculosis* H37Ra (data not shown). By definition, these results show that *M. tuberculosis* H37Rv and H37Ra express PLC and PLD activities. In contrast, in whole-cell extracts from *M. smegmatis* mc^{2}155, only PA was detected upon incubation with phosphatidylcholine, suggesting that this organism produces PLD, but not PLC, activity. PLC and PLD activities were also detected in extracts from *M. bovis* and *M. marinum* 1218R, while only PLD activity could be detected in *M. bovis* BCG extracts, despite the presence of sequences which hybridize to *mpcA* and *mpcB* gene probes (Fig. 5A). Different sources of *M. bovis* BCG cells and DNA were used in the experiments whose results are shown in Fig. 4 and 5A. We verified that the absence of PLC activity in *M. bovis* BCG was not due to strain differences by probing chromosomal DNA isolated from the same *M. bovis*

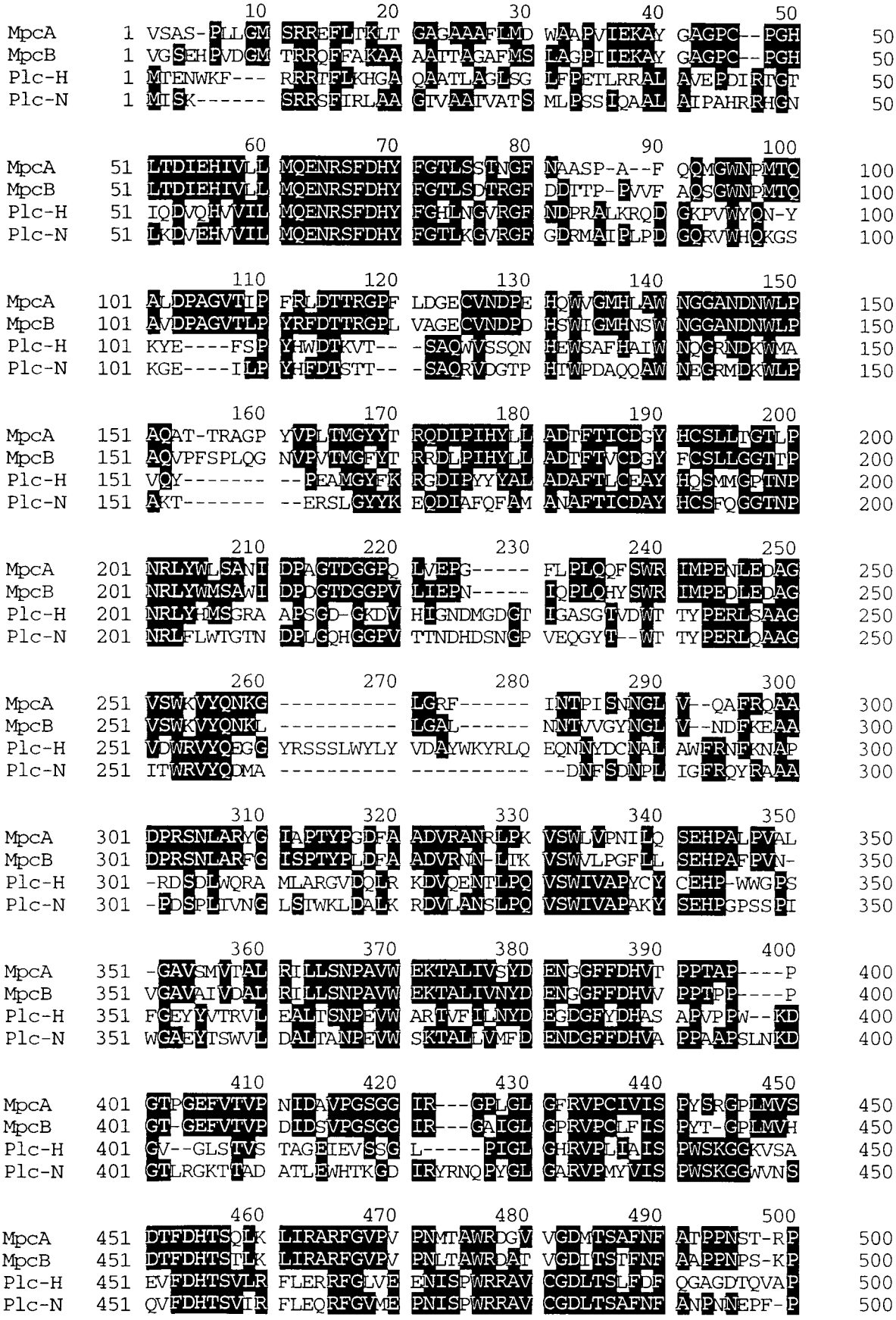

FIG. 3. Alignment of the deduced amino acid sequences of PLC enzymes from *M. tuberculosis* and *P. aeruginosa*. Identical amino acid residues (black highlighting) are indicated. Only the first 500 amino acid residues are shown, as MpcA and MpcB are significantly smaller than PlcH and PlcN and their homology is substantially reduced after this point.

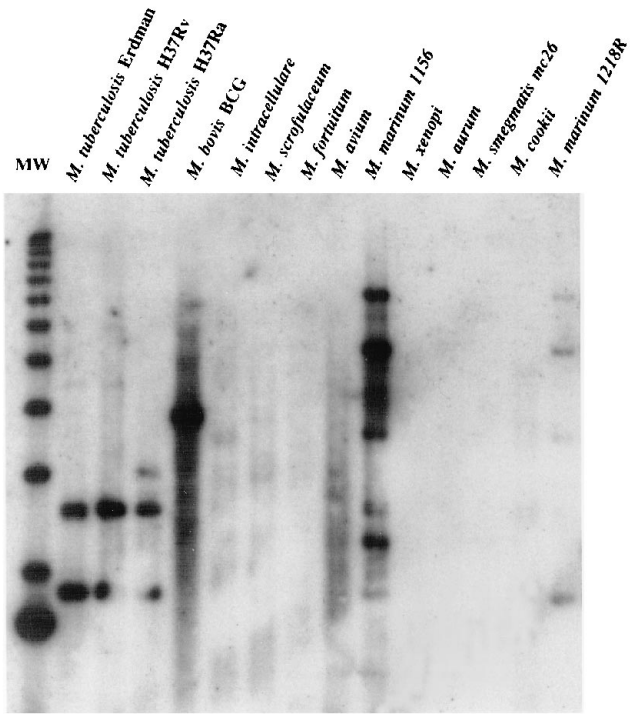

FIG. 4. Southern hybridization using an *mpcB* gene probe. Chromosomal DNAs from the indicated *Mycobacterium* spp. were digested with *Sal*I and probed with a full-length *mpcB* gene probe generated by PCR. Hybridization is seen with *M. tuberculosis* strains, *M. marinum* strains, and *M. bovis* BCG. Identical results are seen with an *mpcA* gene probe, as *mpcA* and *mpcB* cross-hybridize under the hybridization conditions used (see Materials and Methods). MW, molecular weight markers.

BCG cells as those used in the TLC analysis. The results were identical to those shown in Fig. 4 (data not shown). When extracts from *M. smegmatis* mc^{2}155 harboring either pMX-mpcA or pMX-mpcB were analyzed for phospholipase activity, both PA and DAG were detected, indicating that the *mpcA* and *mpcB* genes each encode a functional PLC enzyme (Fig. 5B). In some *M. tuberculosis* H37Rv reactions, a product most likely representing phosphatidylethanol is seen. This product occurs when PA is transferred to a primary alcohol group to form phosphatidylalcohol (13). Neither PLC nor PLD activity could be detected in culture filtrates from *M. tuberculosis* H37Rv or *M. smegmatis* mc^{2}155, suggesting that these enzymes remain cell associated. The deduced amino acid sequences of both MpcA and MpcB have N-terminal regions homologous to the signal sequences of *P. aeruginosa* PlcH and PlcN (Fig. 3). The inability to detect PLC activity in culture filtrates suggests that while MpcA and MpcB may be exported, they are not released into the external milieu. This contention is supported by our finding that PLC activity is still detected when intact cells are used in reaction mixtures (data not shown). To the best of our knowledge, this is the first demonstration of PLC and PLD activity in mycobacteria.

Sphingomyelinase activity in mycobacteria. To determine whether other phospholipids besides phosphatidylcholine can be hydrolyzed by the phospholipase activities found in mycobacteria, we tested sphingomyelin as a substrate. The ability to hydrolyze sphingomyelin is significant, as the generation of ceramide through its breakdown can mimic the effects of TNF-α (26). Table 2 shows the results of sphingomyelinase assays. Sphingomyelinase activity was detected in whole-cell extracts from *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, *M. marinum* 1218R, *M. bovis*, and *M. bovis* BCG, but not from *M. smegmatis* mc^{2}155. However, sphingomyelinase activity was detected when whole-cell extracts from *M. smegmatis* mc^{2}155 carrying either pMX-mpcA or pMX-mpcB were examined. These results show that in addition to hydrolyzing phosphatidylcholine, the gene products of *mpcA* and *mpcB* can also hydrolyze sphingomyelin.

DISCUSSION

In this study, we demonstrate the presence of PLC and PLD activities in various *Mycobacterium* spp. and show that the gene products homologous to PlcH and PlcN of *P. aeruginosa* encode functional PLC enzymes. We were able to obtain the complete nucleotide sequence of *mpcB*, and expression of PLC activity in *M. smegmatis* cells harboring the recombinant gene supports our assignment of initiation signals. Unlike the PLC enzymes from *P. aeruginosa*, which are secreted into the external milieu, the PLC enzymes from *M. tuberculosis* appear to remain cell associated, as we were unable to detect PLC or PLD activity in culture filtrates from *M. tuberculosis* H37Rv or *M. smegmatis* mc^{2}155 but could still detect activity when intact cells were used.

The high degree of nucleotide identity and the tandem arrangement of the *mpcA* and *mpcB* genes suggest that one or the other may have arisen through a gene duplication event. We can only speculate what the role of two such highly similar genes may be in the biology or pathogenesis of *M. tuberculosis*. As noted earlier, other organisms, such as *L. monocytogenes* and *P. aeruginosa*, also have two PLC genes. In these organisms, the PLC enzymes have distinct substrate preferences. Further analysis of the PLC enzymes from *M. tuberculosis* may reveal that, while they share phosphatidylcholine and sphingomyelin as substrates, they may also have different, nonoverlapping substrates. Similar to the different roles of the *L. monocytogenes* PLCs in escape from phagocytic vesicles, the two PLCs of *M. tuberculosis* may play discrete roles in pathogenesis. It may also be that the *mpcA* and *mpcB* genes are regulated differently and act at distinct stages during infection.

Southern hybridization studies showed that other *Mycobacterium* spp. contain sequences homologous to *mpcA* and *mpcB*. These results are in contrast to earlier reports suggesting that *mpcA* and *mpcB* are unique to *M. tuberculosis*, and they decrease the utility of *mpcA*- or *mpcB*-specific PCR or hybridization as a diagnostic tool (3). Examination of whole-cell extracts from *M. marinum* 1218R and *M. bovis* showed that they contain PLC enzyme activity. The presence of hybridizing sequences strongly suggests that the PLC enzyme(s) present in these organisms is homologous to those from *M. tuberculosis*. However, it is not possible to determine from this assay whether one PLC or both PLC enzymes are functional in these species or if additional PLC enzymes are responsible for the activity we observed.

We were not able to detect PLC activity in whole-cell extracts from *M. bovis* BCG, even upon prolonged exposure of the autoradiogram, despite the presence of hybridizing sequences in this organism. This finding suggests that there may be a defect in the *mpcA* and/or *mpcB* gene of *M. bovis* BCG. Such a defect could include a point mutation, a regulatory mutation, or a defect in export of the protein. In contrast, PLC and PLD activities were detected in the avirulent *M. tuberculosis* strain H37Ra, a derivative of H37Rv. The genetic differences between these strains are not clear, although a recent report suggests that they may be point mutations or small deletions (17). Such differences could affect regulatory genes controlling expression of *mpcA* and *mpcB*. Environmental reg-

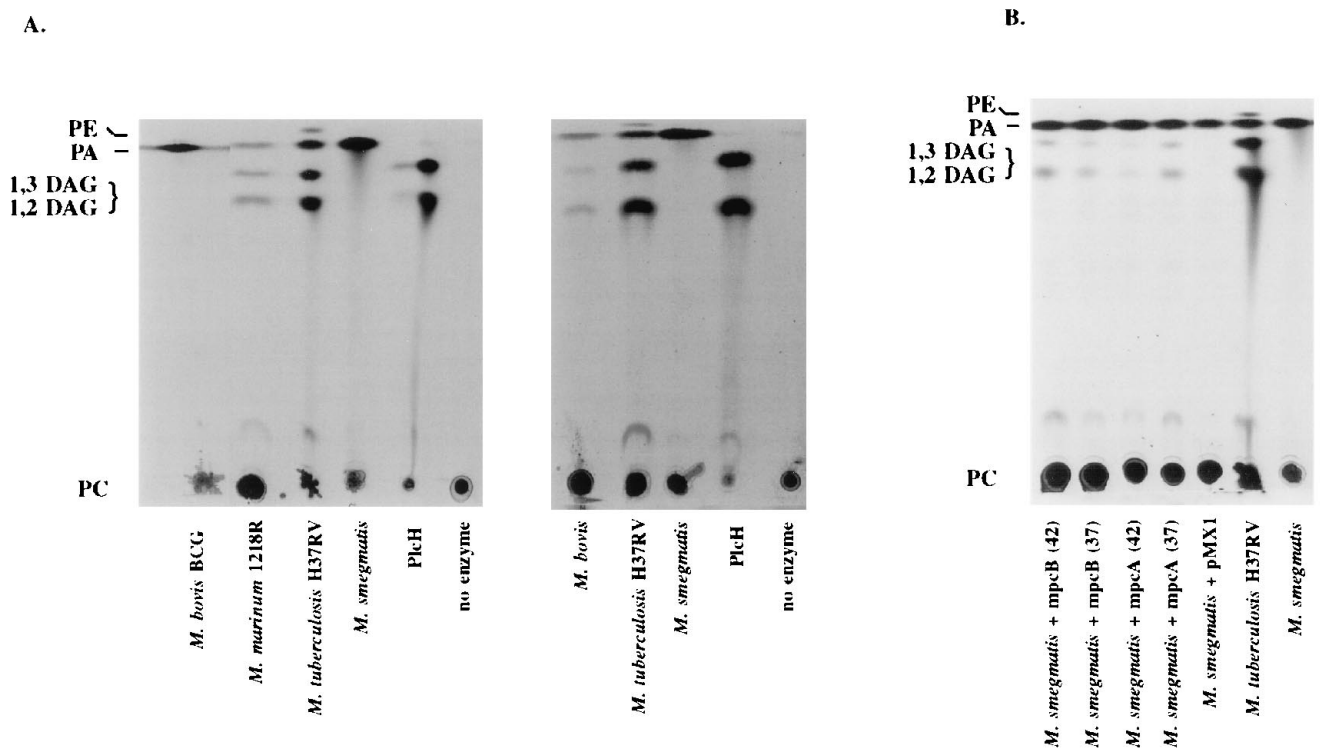

FIG. 5. TLC analysis of lipid hydrolysis products. (A) Results of phospholipid hydrolysis using whole-cell extracts from the indicated *Mycobacterium* spp. and purified PlcH from *P. aeruginosa*. (B) Expression of PLC activity in *M. smegmatis* harboring recombinant *mpcA* and *mpcB*. Whole-cell extracts from *M. smegmatis* containing pMX-mpcA, pMX-mpcB, or pMX1 and grown at 37 or 42°C were analyzed. Note that chain migration of the labeled fatty acid moiety results in two labeled species of DAG. PC, phosphatidylcholine; PE, phosphatidic ethanol.

ulation of PLC and PLD activities in *M. tuberculosis* has not yet been examined. Alternatively, other virulence factors which may act at an earlier stage in infection or the disease process may be affected in H37Ra, and infection by this strain may be controlled prior to the stage at which the PLC enzymes act. PLC was also not detected in *M. smegmatis* mc^{2}155. However, this organism lacks PLC hybridizing sequences.

Sphingomyelinase activity was found in *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, *M. marinum* 1218R, *M. bovis*, and *M. bovis* BCG but not in *M. smegmatis* mc^{2}155. This activity could be expressed in *M. smegmatis* mc^{2}155 carrying either *mpcA* or *mpcB*, indicating that both gene products encode PLC enzymes which can hydrolyze sphingomyelin. This finding is in contrast to what is observed with the PLC enzymes from *P. aeruginosa*, of which PlcH, but not PlcN, is able to hydrolyze sphingomyelin. Sphingomyelinase activity seen in *M. bovis* BCG is probably due to its PLD, as PLC activity was not detected in this strain.

PLD activity was found in every species of *Mycobacterium* examined, suggesting that it may be important in the biology of this genus. It is not possible a priori to predict whether the genes encoding these enzymes are similar in different mycobacteria or if more than one PLD enzyme is present. The PLD activity from *M. tuberculosis* H37Rv may be a phosphatidylcholine-specific PLD because of the phosphatidylalcohol product observed in TLC analysis. To date, only phosphatidylcholine-specific PLDs have been shown to catalyze the transfer of the phosphatidyl group to a primary alcohol (13). We did not observe the formation of phosphatidylalcohol in reactions using whole-cell extracts from other mycobacteria, suggesting that the PLD enzymes from these organisms may have a broader substrate specificity. We are currently undertaking the purification of PLD enzymes in order to characterize them further.

Identification of PLC activity may be relevant to *M. tuberculosis* pathogenesis at several levels. First, the generation of DAG from PC hydrolysis may alter cell signaling events through protein kinase C activation. Such alterations could conceivably affect macrophage activation states, or intracellular trafficking by turning on protein kinase C targets at inappropriate times. Second, the inflammatory response could be amplified through the production of downstream metabolites of DAG, such as eicosinoids. Finally, PLC may be cytotoxic or induce apoptosis, as has been observed with PlcH from *P. aeruginosa* (2). Some toxicity of *M. tuberculosis* for monocytes has been observed previously (28).

TABLE 2. Sphingomyelinase activity in mycobacteria

Strain(s) or species	Sphingomyelinase activity[a]
M. tuberculosis H37Rv, H37Ra	+
M. smegmatis mc^{2}155	−
M. bovis	+
M. bovis BCG	+
M. marinum 1218R	+
M. smegmatis + pMX1	−
M. smegmatis + pMX-mpcA	+
M. smegmatis + pMX-mpcB	+

[a] +, presence of sphingomyelinase activity as defined in Materials and Methods; −, absence of detectable activity.

MpcA and MpcB were also found to have sphingomyelinase activity. Through the generation of ceramide by hydrolysis of sphingomyelin, the effects of TNF-α can be mimicked. It is possible that sphingomyelinase production within macrophages can contribute to their death, as it has been observed that macrophages containing *M. tuberculosis* are exquisitely sensitive to killing by TNF-α (5, 6).

PLD activity may also contribute to pathogenesis by the generation of intracellular signaling molecules or by cytotoxicity. PLD is a virulence factor of *C. pseudotuberculosis* (10, 18) and also the toxic component of brown recluse spider venom (15). PA, the lipid product of phosphatidylcholine hydrolysis by PLD, has been shown to be an effector molecule in a number of physiological events, such as DNA synthesis (21), cell proliferation (36), and secretion (31). Moreover, PA can be converted to DAG and other second messengers and could potentially exacerbate the effects of PLC activity.

The identification of PLC and PLD activity in *M. tuberculosis* is significant in the potential for these enzymes to impact pathogenesis. Further characterization of PLC and PLD substrate specificity and their expression in vivo has the potential to increase our understanding of the survival and disease-causing mechanism of this ancient pathogen.

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