INFECTION AND IMMUNITY, Nov. 1995, p. 4301–4306 Vol. 63, No. 11
0019-9567/95/$04.00+0
Copyright © 1995, American Society for Microbiology

A Species-Specific Nucleotide Sequence of *Mycobacterium tuberculosis* Encodes a Protein That Exhibits Hemolytic Activity when Expressed in *Escherichia coli*

SYLVIA CARDOSO LEÃO,* CLAUDIA LUCIA ROCHA, LUIS ANGEL MURILLO, CARLOS ALBERTO PARRA,† AND MANUEL ELKIN PATARROYO

Instituto de Inmunología-Hospital San Juan de Dios, Universidad Nacional de Colombia, Bogotá, Colombia

Received 21 February 1995/Returned for modification 14 June 1995/Accepted 17 August 1995

Species-specific proteins may be implicated in the unique pathogenic mechanisms characteristic of *Mycobacterium tuberculosis*. In previous studies, a 3.0-kb species-specific DNA fragment of *M. tuberculosis* was identified (C. A. Parra, L. P. Londoño, P. del Portillo, and M. E. Patarroyo, Infect. Immun. 59:3411–3417, 1991). The nucleotide sequence of this 3.0-kb fragment has been obtained. This sequence was shown to contain two open reading frames (ORFs) whose putative gene products share 68.9% identity between each other. The major ORF shows 57.8% similarity with PLC-N and 53.2% similarity with PLC-H, two phospholipase C enzymes from *Pseudomonas aeruginosa*. The major ORF was amplified by PCR and cloned into the pGEX-5T expression vector. Cell extracts of *Escherichia coli* overexpressing this glutathione *S*-transferase fusion protein were shown to produce beta-hemolysis suggestive of phospholipase activity. Since phospholipase C enzymes have been reported as virulence factors of *P. aeruginosa* and also of the intracellular pathogen *Listeria monocytogenes*, it is possible that the proteins identified in this study could also play a role in sustaining tuberculosis infection in humans.

Tuberculosis is by far the most prevalent infectious disease worldwide. In underdeveloped countries, tuberculosis has never ceased to be a major health problem. The health problem is now worsening in developed countries, where the disease was thought to be under control, as numbers of *Mycobacterium tuberculosis* isolates with multidrug resistance continue to rise (33). The magnitude of the problem has increased dramatically during the past decade, mainly as a result of migration and inner city poverty and the association of tuberculosis with human immunodeficiency virus infection.

The following practical obstacles to controlling tuberculosis are well known: the *Mycobacterium bovis* BCG vaccine shows variable results with regard to protection (14), the diagnosis of tuberculosis still relies upon direct microscopic visualization of bacilli in clinical samples or in culture, both unsatisfactory methods for confirmation of suspected extrapulmonary or childhood tuberculosis, and the treatment of the disease requires compliance with prolonged multidrug therapeutic schemes. Since the recent increase in cases of tuberculosis, considerable attention has been devoted to the development of improved drugs and vaccines against the disease as well as diagnostic methods with greater sensitivity and specificity.

Part of the solution to better control tuberculosis may rely on a better understanding of the biology of the infection of mammalian cells by the bacilli. To produce disease, successful intracellular pathogens like *M. tuberculosis* must evade the host cell's mechanisms of killing. Different strategies of these pathogens seem to contribute to sustain an infection inside the cell. They include inhibition of lysosome fusion (3, 17), resistance to the lysosomal contents (18), resistance to cationic peptides (13), modification of the phagolysosome to facilitate survival and multiplication of the parasite (31), and escape of the pathogen from the phagolysosome into the cytoplasm (9). It is unclear which strategies *M. tuberculosis* uses to survive inside cells. Recent contributions to this field are the demonstration that the vacuoles containing *M. avium* fail to acidify below pH 6.5 (35), the identification of a DNA fragment that may allow the pathogen to invade and survive inside human cells (4), and experimental evidence that viable tubercle bacilli may have the capacity to escape from the phagolysosome (23).

In studies designed to aid in the development of a synthetic vaccine against tuberculosis, species-specific proteins unique to *M. tuberculosis* were identified and studied (21). As an outcome of this research strategy, a species-specific DNA fragment of *M. tuberculosis*, designated *mtp40*, was cloned and sequenced (25). *mtp40* is a 266-bp fragment contained on a 3.0-kb *Bam*HI fragment that hybridized only to *M. tuberculosis* DNA and not to DNA from other mycobacterial species, including components of the *M. tuberculosis* complex, *M. bovis*, and *M. bovis* BCG. The complete nucleotide sequence of this unique 3.0-kb fragment is reported here. Two open reading frames (ORFs) identified in this sequence have significant similarity at the nucleotide and amino acid levels to two phospholipase C enzymes (PLCs) from *Pseudomonas aeruginosa*. The relevance that these proteins may have in the course of infection of eukaryotic cells by *M. tuberculosis* is discussed.

MATERIALS AND METHODS

DNA sequencing. A Bluescript plasmid vector containing the 3.0-kb *Bam*HI fragment of *M. tuberculosis* H37Rv DNA (25) was double digested with *Bam*HI and *Eco*RI, releasing three fragments of 1,544, 261, and 1,212 bp. The 1.5- and 1.2-kb *Bam*HI-*Eco*RI fragments corresponded to the flanking regions of the *mtp40* fragment (25). These fragments were electroeluted from a 1% agarose gel and separately cloned into *Eco*RI-*Bam*HI-digested Bluescript KS phagemid

* Corresponding author. Present address: Universidade Federal de São Paulo-Escola Paulista de Medicina, Rua Botucatu, 862 3° andar 04023-062 São Paulo, SP Brazil. Phone: (55-11) 549-8210. Fax: (55-11) 571-1095.

† Present address: Washington University School of Medicine, Department of Pathology, St. Louis, MO 63110.

(Stratagene, La Jolla, Calif.). *Escherichia coli* DH5α competent cells were transformed with the recombinant phagemids.

DNA sequencing of the 1.5- and 1.1-kb *Bam*HI-*Eco*RI fragments was carried out by the dideoxynucleotide chain termination method (30), with the Sequenase kit (U.S. Biochemicals) and α-^{35}S-dATP (Amersham). The double-stranded DNA was previously denatured with alkali. Nested deletions of the 1.5-kb fragment produced with a commercial kit (Erase-a-Base; Promega) and synthetic oligonucleotide primers were used in the sequencing strategy. The sequences from the 1.5- and 1.1-kb fragments were aligned with the previously obtained sequence of *mtp40*. Computer analysis was performed with the following software packages: GCG (University of Wisconsin Biotechnology Center, Madison) and Geneworks (Intelligenetics). Protein similarities were searched in databases (GenBank release 73) by use of the programs TFASTA (average match, 0.54; average mismatch, −0.396) and BLAST. Optimal alignment of protein sequences (26) was obtained with the program PILEUP (gap weight, 3.0; gap length weight, 0.1).

Primers and amplification by PCR. One set of primers was synthesized and used in the PCR amplification of a fragment of 1,585 bp from the region containing the major ORF (HL-A). To date, in sequences reported for mycobacterial genes, GUG is used as a start codon nearly as frequently as AUG (11). Therefore, the primers were designed to amplify the coding region, assuming the GUG residue at position 438 is the initiation codon. The sequences of the primers were modified to generate *Bam*HI restriction sites. The sequences, with the modified nucleotides underlined, were: 5′GCAAGGATCCGCAAGCCCA 3′ (433 to 451) and 5′GGGAGGATCCGGGAAATT 3′ (positions 2019 to 2001). Fifty-microliter reaction mixtures containing 100 ng of purified *M. tuberculosis* DNA, 20 pmol of each primer, 100 μM each deoxynucleoside triphosphate, 5 U of *Taq* polymerase, and 5 μl of 10× PCR buffer (100 mM Tris-HCl [pH 8.3], 500 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin) (Perkin-Elmer Cetus) were subjected to PCR amplification. To compensate for the mismatches generated in the primers by the creation of the restriction sites, the 10 initial cycles of the PCR were performed at a lower temperature of annealing. Therefore, an initial denaturation at 95°C for 5 min was followed by 10 cycles at an annealing temperature of 48°C for 1 min, an extension temperature of 72°C for 2 min, and a denaturing temperature of 95°C for 1 min. Afterwards, 25 cycles were performed with a higher annealing temperature of 60°C. The final extension step was performed at 72°C for 5 min. The PCR product was purified with Geneclean (Bio 101). Two positive amplification controls were used to corroborate the result of this PCR by subjecting the amplified fragment to reamplification with two sets of internal primers (data not shown).

Expression of the major ORF. The expression vector pGEX-5T (Pharmacia) (8) and the 1,585-bp fragment amplified by PCR were digested with *Bam*HI and ligated. *E. coli* XL1 Blue was transformed with the ligation product, and the recombinants containing the insert in the correct position were identified after digestion with *Bam*HI and *Eco*RI. Overnight cultures of *E. coli* XL1 Blue transformed with the nonrecombinant and recombinant pGEX-5T plasmids were diluted 1:10 and grown for 2 h at 37°C before adding 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside; Bio-Rad). After an additional 4 h of growth at 37°C, the cells were pelleted, resuspended in 200 μl of Laemmli buffer, and boiled for 10 min. Ten microliters of each pellet was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% acrylamide gels, and proteins were visualized by staining with Coomassie blue.

Hemolysis assay. Overnight cultures of *E. coli* XL1 Blue transformed with the recombinant and nonrecombinant clones and induced with 0.1 mM IPTG were pelleted, resuspended in 0.1 volume of distilled water, and sonicated. Ten microliters of each sonic extract was applied to blood-agar plates (blood agar base [Oxoid, Hampshire, England] supplemented with 5% defibrinated sheep erythrocytes and 10 mM $CaCl_2$), and the plates were incubated at 37°C for 24 h. Noninduced transformants were used as a control of hemolysis.

GenBank accession number. The nucleotide sequence for the 3.0-kb fragment has been included in the GenBank database under the accession number L11868.

RESULTS AND DISCUSSION

The complete nucleotide sequence of the 3.0-kb fragment was obtained after sequencing the 1.5- and 1.2-kb fragments with nested deletions and synthetic primers by the strategy described in Materials and Methods. The nucleotide sequence of the central 266-bp fragment was reported previously (25). The complete sequence of the 3.0-kb fragment is shown in Fig. 1. It comprises 3,015 bp with a G+C content of 62.1% and two putative ORFs. The major ORF (HL-A) starts at nucleotide 414 and ends after 1,584 bp, with a TGA on nucleotide 1998. A second ORF (HL-B) starts at nucleotide 2222 and remains open beyond the 3′ end of the 3.0-kb fragment. A total of 1,004 bp of this ORF have been sequenced. The codon usage in both ORFs is in accordance with the codon preference observed for other *M. tuberculosis* genes (11). The predicted molecular weight of HL-A is 56,949, and the isoelectric point is 6.57. The hydrophobic-hydrophilic profile demonstrates that, except for the leader sequence in the N terminus, the protein is highly hydrophilic (data not shown). The 38 N-terminal amino acids of HL-A resemble a signal sequence, with charged amino acids within the first 10 residues, followed by a core of at least 9 hydrophobic residues (sufficient to span the membrane), followed at the end by a helix-breaking residue (proline) within 4 to 8 residues preceding the cleaving site, which very frequently is an alanine (39). Upstream of the initiation codon of HL-A there is a sequence, shown in bold in Fig. 1, that is similar to the Pho-box sequence located upstream of the *phoA* gene in *E. coli* (32).

The analysis of the DNA sequence obtained from the FASTA program revealed a significant degree of similarity between the two ORFs of the *M. tuberculosis* 3.0-kb fragment and two genes of *P. aeruginosa*, *plcN* and *plcS*, which encode the nonhemolytic (PLC-N) and the hemolytic (PLC-H) PLCs, respectively (24, 28). The DNA sequence of the major ORF (HL-A) is 63% identical to *plcN* and 55.9% identical to *plcS*. The analysis also revealed 75% identity between the 5′ region of the major ORF and the second ORF on the *M. tuberculosis* 3.0-kb fragment.

The analysis of the amino acid sequences of these ORFs by use of the TFASTA and BLAST programs also demonstrated a high degree of identity and similarity to sequences of the two PLCs of *P. aeruginosa*. HL-A from *M. tuberculosis* has 39.7% identity and 57.8% similarity to PLC-N from *P. aeruginosa*. HL-A has a high degree of similarity to PLC-H from *P. aeruginosa* as well; they are 33.3% identical and 53.2% similar. HL-A is nearly 25% shorter than PLC-H (731 amino acids) and PLC-N (693 amino acids). The highest degree of similarity between these proteins is located towards the N terminus (first two-thirds of the proteins), whereas the C terminus is less conserved. The second ORF encodes a putative polypeptide which is truncated at 334 amino acids (Fig. 1, HL-B). Its amino acid sequence is 68.9% identical to the 334 N-terminal amino acids of HL-A. It is 37.6% identical and 55.6% similar to the N-terminal sequence of PLC-H of *P. aeruginosa*. The comparison of the amino acid sequences of the polypeptides encoded by the two ORFs of the 3.0-kb fragment with the sequences of the two PLCs of *P. aeruginosa* is shown in Fig. 2.

The similarity of the proteins coded for in the identified ORFs with the PLCs suggests that these putative *M. tuberculosis* proteins could also have similar activities. To test this hypothesis, a fragment containing the region of the HL-A ORF was amplified by PCR and cloned into the pGEX-5T expression vector. The recombinant clone containing the insert in the correct sense expressed a protein of 83 kDa after induction with IPTG corresponding to the 27-kDa glutathione *S*-transferase plus the 56-kDa expected molecular mass of the recombinant protein (Fig. 3). Clones bearing the insert in the opposite sense produced only the 27-kDa glutathione *S*-transferase upon induction with IPTG (data not shown).

The recombinant clone was beta-hemolytic in blood-agar plates after induction with IPTG (Fig. 4). Noninduced recombinants as well as induced transformants of pGEX-5T without the insert were nonhemolytic. PLCs from *Bacillus cereus*, *Clostridium perfringens*, and *P. aeruginosa* are hemolytic in similar in vitro assays (16, 36, 37). The virulent *M. tuberculosis* strain H37Rv and to a lesser extent the avirulent strain H37Ra, but not *M. bovis* BCG, have been shown to display contact-dependent cytolytic activity in an erythrocyte lysis assay (20). King et al. (20) isolated a ~3.2-kb fragment from an *M. tuberculosis* cosmid library which conferred contact-dependent cytolysis to *E. coli*. It is important to note that a comparison of the partial

```
   1 GGATCCGACGGTGTCAGCCGTCGCCACCAGAGTCAACATCAGCACCGTAGTCCACATGGTACGGACGCTGCATTGGCGGCGCGGTTGGGCAGATACGAAA
 101 TTAGTTTACCGATAGCGCGATATCCGTCGCTGACCTCGAACTGTCTAGTGACTTAAGCGCCGAAGCCGGCCGGTGAGATCGGTCATATCAAGTTTTGTCA
 201 GGAATGCGGGATTCGAATAATTTTTTACTGTCGGCGAGTGCCCGATACCCAGCGACAATCATGGTTACGGCACCCTTCCCGCTGCGATATCCCCTAGACG
 301 GCCGTCGGCCGCGCCAAACGGTGCGGTTGCGGTTGCTGGAACGCGAAAATCCGCGTGCGACACGCGGTTGGCGTATCGCGATGGACCGCTGTCGTCACTA
                                                 *
      HL-A A  E  C  R  K  A  G  Q  V  S  A  S  P  L  L  G  M  S  R  R  E  F  L  T  K  L  T  G  A
 401 TAACGTGATCTGAGCTGAGTGCAGGAAGGCAGGGCAAGTGAGCGCAAGCCCACTCCTCGGAATGTCACGTCGAGAGTTTTTGACAAAGCTCACTGGCGCA

     G  A  A  A  F  L  M  D  W  A  A  P  V  I  E  K  A  Y  G  A  G  P  C  P  G  H  L  T  D  I  E  H  I  V
 501 GGCGCAGCGGCATTCCTGATGGACTGGGCTGCACCGGTGATTGAAAAGGCCTACGGCGCCGGGCCTTGTCCCGGACATTTGACCGACATCGAGCATATCG

      L  L  M  Q  E  N  R  S  F  D  H  Y  F  G  T  L  S  S  T  N  G  F  N  A  A  S  P  A  F  Q  Q  M  G
 601 TGTTGCTGATGCAGGAGAACCGGTCATTCGACCACTATTTCGGAACGCTTTCCAGCACCAATGGGTTCAACGCCGCGTCGCCGGCATTCCAACAAATGGG

      W  N  P  M  T  Q  A  L  D  P  A  G  V  T  I  P  F  R  L  D  T  T  R  G  P  F  L  D  G  E  C  V  N
 701 TTGGAACCCCATGACGCAGGCGTTGGACCCCGCCGGGGTCACCATTCCGTTCCGCTTGGACACCACCCGAGGCCCCTTCCTGGACGGCGAGTGCGTCAAC

     D  P  E  H  Q  W  V  G  M  H  L  A  W  N  G  G  A  N  D  N  W  L  P  A  Q  A  T  T  R  A  G  P  Y  V
 801 GACCCCGAGCACCAGTGGGTGGGGATGCACCTGGCCTGGAACGGTGGTGCCAACGACAACTGGCTGCCGGCGCAGGCGACCACCCGCGCAGGACCATATG

      P  L  T  M  G  Y  Y  T  R  Q  D  I  P  I  H  Y  L  L  A  D  T  F  T  I  C  D  G  Y  H  C  S  L  L
 901 TCCCTTTGACCATGGGTTACTACACGCGCCAAGACATCCCGATCCACTATCTGCTGGCCGACACGTTCACCATCTGCGACGGCTACCATTGCTCGCTGCT

      T  G  T  L  P  N  R  L  Y  W  L  S  A  N  I  D  P  A  G  T  D  G  G  P  Q  L  V  E  P  G  F  L  P
1001 GACGGGCACCCTGCCCAACCGGCTCTACTGGTTGAGCGCCAACATCGACCCCGCCGGCACCGACGGGGGACCCCAATTGGTAGAGCCGGGCTTCCTGCCG

     L  Q  Q  F  S  W  R  I  M  P  E  N  L  E  D  A  G  V  S  W  K  V  Y  Q  N  K  G  L  G  R  F  I  N  T
1101 CTGCAGCAATTCAGTTGGCGCATCATGCCGGAAAACCTCGAAGATGCCGGGGTCAGCTGGAAGGTGTACCAGAACAAGGGCCTCGGGCGATTCATCAACA

      P  I  S  N  N  G  L  V  Q  A  F  R  Q  A  A  D  P  R  S  N  L  A  R  Y  G  I  A  P  T  Y  P  G  D
1201 CGCCCATCAGCAATAACGGGCTGGTGCAGGCCTTCCGCCAGGCAGCTGATCCGAGGTCGAACTTGGCCCGCTACGGTATCGCCCCGACCTACCCTGGGGA

      F  A  A  D  V  R  A  N  R  L  P  K  V  S  W  L  V  P  N  I  L  Q  S  E  H  P  A  L  P  V  A  L  G
1301 CTTCGCTGCCGACGTCAGGGCCAACCGGCTACCCAAGGTCTCCTGGTTAGTTCCCAACATCCTGCAGTCCGAACACCCCGCCCTGCCGGTAGCGCTTGGC

     A  V  S  M  V  T  A  L  R  I  L  L  S  N  P  A  V  W  E  K  T  A  L  I  V  S  Y  D  E  N  G  G  F  F
1401 GCGGTGTCCATGGTGACCGCGCTGCGGATCTTGCTGTCCAATCCCGCGGTGTGGGAAAAGACCGCACTTATCGTCAGCTATGACGAGAACGGCGGCTTCT

      D  H  V  T  P  P  T  A  P  P  G  T  P  G  E  F  V  T  V  P  N  I  D  A  V  P  G  S  G  G  I  R  G
1501 TCGACCACGTCACGCCCCCCACGGCACCGCCCGGGACACCCGGCGAATTCGTCACGGTGCCCAACATCGACGCAGTACCCGGGTCCGGTGGCATTCGTGG

      P  L  G  L  G  F  R  V  P  C  I  V  I  S  P  Y  S  R  G  P  L  M  V  S  D  T  F  D  H  T  S  Q  L
1601 TCCGCTCGGTCTGGGTTTTCGCGTTCCCTGCATTGTCATTTCGCCGTACAGCCGCGGCCCGCTGATGGTCTCCGACACGTTCGACCACACCTCGCAATTG

     K  L  I  R  A  R  F  G  V  P  V  P  N  M  T  A  W  R  D  G  V  V  G  D  M  T  S  A  F  N  F  A  T  P
1701 AAGTTGATTCGCGCCCGGTTCGGCGTGCCGGTTCCCAACATGACCGCCTGGCGCGACGGCGTGGTTGGCGACATGACCTCAGCGTTCAACTTTGCGACTC

      P  N  S  T  R  P  N  L  S  H  P  L  L  G  A  L  P  K  L  P  Q  C  I  P  N  V  V  L  G  T  T  D  G
1801 CACCGAATTCGACCAGACCCAACTTGAGCCACCCGTTGCTGGGAGCGCTGCCGAAGCTGCCGCAGTGCATCCCTAACGTGGTGTTGGGAACCACCGACGG

      A  L  P  S  I  P  Y  R  V  P  Y  P  Q  V  M  P  T  Q  E  T  T  P  V  R  G  T  P  S  G  L  C  S  *
1901 CGCGTTGCCGAGCATTCCCTATCGGGTGCCCTATCCGCAGGTGATGCCAACTCAGGAAACCACACCCGTCCGCGGGACTCCCAGCGGGCTGTGCAGCTGA

      HL-B  S  P  G  V  R  F  A  C  M  C  A  S  K  S  P  A  P  I  P  R  P  P  R  Q  A  G  G  I  R
2001 AATTTCCCGGTAATCTCCCGGCGTTCGATTTGCTTGCATGTGCGCGAGCAAATCGCCCGCACCAATCCCGCGCCCGCCCCGGCAGGCAGGCGGAATCAGA

     T  R  H  A  G  R  Q  G  I  A  D  R  G  R  N  Y  T  V  I  S  N  G  R  V  G  R  Q  G  G  R  V  G  S  E
2101 ACACGACACGCCGGTCGGCAAGGCATAGCCGATCGCGGCCGCAACTATACCGTGATCAGCAATGGCCGTGTGGGCCGTCAAGGAGGGCGCGTGGGGAGCG

      H  P  V  D  G  M  T  R  R  Q  F  F  A  K  A  A  A  A  T  T  A  G  A  F  M  S  L  A  G  P  I  I  E
2201 AACACCCCGTCGACGGAATGACCCGCCGACAATTTTTTGCCAAAGCCGCCGCCGCTACCACCGCCGGGGCCTTCATGTCCTTGGCTGGTCCGATTATCGA

      K  A  Y  G  A  G  P  C  P  G  H  L  T  D  I  E  H  I  V  L  L  M  Q  E  N  R  S  F  D  H  Y  F  G
2301 AAAAGCCTACGGAGCGGGGCCTTGCCCCGGACATTTGACCGACATCGAGCACATCGTGTTGTTGATGCAGGAGAATCGGTCATTCGATCACTACTTCGGC

     T  L  S  D  T  R  G  F  D  D  T  T  P  P  V  V  F  A  Q  G  G  W  N  P  M  T  Q  A  V  D  P  A  G  V
2401 ACTCTTTCTGACACCCGCGGGTTCGATGACACCACCCCGCCGGTGGTATTCGCGCAGTCGGGCTGGAACCCGATGACACAGGCGGTTGACCCCGCCGGCG

      T  L  P  Y  R  F  D  T  T  R  G  P  L  V  A  G  E  C  V  N  D  P  D  H  S  W  I  G  M  H  N  S  W
2501 TCACCCTTCCATATCGCTTCGACACCACCCGGGGCCCGCTGGTCGCCGGCGAATGCGTCAACGACCCGGACCACAGCTGGATCGGCATGCACAACTCGTG

      N  G  G  A  N  D  N  W  L  P  A  Q  V  P  F  S  P  L  Q  G  N  V  P  V  T  M  G  F  Y  T  R  R  D
2601 GAACGGCGGCGCCAACGACAACTGGCTGCCGGCGCAGGTCCCGTTCAGTCCGTTGCAGGGCAACGTGCCGGTCACGATGGGTTTCTACACGCGTCGTGAC

     L  P  I  H  Y  L  L  A  D  T  F  T  V  C  D  G  Y  F  C  S  L  L  G  G  T  T  P  N  R  L  Y  W  M  S
2701 CTGCCCATTCACTACCTGCTAGCCGACACGTTCACGGTCTGCGACGGCTATTTTTGCTCGCTGCTGGGCGGGACCACGCCCAACCGGCTCTACTGGATGA

      A  W  I  D  P  D  G  T  D  G  G  P  V  L  I  E  P  N  I  Q  P  L  Q  H  Y  S  W  R  I  M  P  E  N
2801 GCGCCTGGATCGACCCCGACGGCACTGACGGCGGGCCGGTGCTGATCGAGCCCAATATCCAACCTCTGCAGCACTACAGCTGGCGCATCATGCCGGAGAA

      L  E  D  A  G  V  S  W  K  V  Y  Q  N  K  L  L  G  A  L  N  N  T  V  V  G  Y  N  G  L  V  N  D  F
2901 CCTCGAAGATGCCGGGGTCAGCTGGAAGGTGTACCAAAACAAATTGCTGGGGGCTCTCAACAACACCGTCGTCGGCTACAACGGGCTGGTCAATGACTTC

     K  Q  A  A  D
3001 AAGCAGGCCGCGGATCC
```

FIG. 1. Complete nucleotide sequence of the 3.0-kb fragment and amino acid sequences deduced from the regions corresponding to the two identified ORFs. A putative valine (GUG) initiation codon at position 438 is indicated with an asterisk. A sequence that matches 9 of 18 nucleotides with a Pho-box is shown in bold type. The *Eco*RI restriction sites corresponding to the limits of the 266-bp fragment described in reference 25 are underlined. Residues of the putative leader peptide in HL-A are underlined.

restriction map of this ~3.2-kb fragment with the restriction map of the 3.0-kb fragment shows that they are not related. It is possible that the product of the recombinant clone pGEX-5T could be activating a cryptic hemolysin in *E. coli* XL1 Blue, as has been demonstrated for the hemolysin inducers HlyX from *Actinobacillus pleuropneumoniae* and BTR from *Bordetella pertussis*. These proteins do not encode hemolysins per se but rather seem to induce a latent hemolysin on a variety of *E. coli* K-12 strains (5, 34). Nevertheless, two important features of this family of proteins suggest that the HL-A protein is not a hemolysin inducer. First, HlyX and BTR are highly homologous to each other and to FNR, the transcriptional regulator of *E. coli*, whereas HL-A is not. Second, unlike HL-A, which has a significant degree of similarity to two PLCs

```
       1                                                  50
HL-A   .......... .......... .......... .......... ..........
HL-B   SPGVRFACMC ASKSPAPIPR PPRQAGGIRT RHAGRQGIAD RGRNYTVISN
Plc-N  .......... .......... .......... .......... ..........
Plc-H  .......... .......... .......... .......... ..........

       51                                                100
HL-A   .AECRKAGQV .SASPLLGMS RREFLTKLTG AGAAAFLMDW AAPVIEKAYG
HL-B   GRVGRQGGRV GSEHPVDGMT RRQFFAKAAA ATTAGAFMSL AGPIIEKAYG
Plc-N  .......... ..MISKS... RRSFIRLAAG TVAATVATSM LPSSIQAALA
Plc-H  .......... ..MTENWKFR RRTFLKHGAQ AATLAGLSGL FPETLRRALA

       101                                               150
HL-A   AGP..CPGHL TDIEHIVLLM QENRSFDHYF GTLSSTNGFN AASPA..FQQ
HL-B   AGP..CPGHL TDIEHIVLLM QENRSFDHYF GTLSDTRGFD DTTPPVVFAQ
Plc-N  IPAHRRHGNL KDVEHVVILM QENRSFDHYF GTLKGVRGFG D...RMAIPL
Plc-H  VEPDIRTGTI QDVQHVVILM QENRSFDHYF GHLNGVRGFN D...PRALKR

       151                                               200
HL-A   MGWNPMTQAL DPAGVTIPFR LDTTRGPFLD GECVNDPEHQ WVGMHLAWNG
HL-B   SGWNPMTQAV DPAGVTLPYR FDTTRGPLVA GECVNDPDHS WIGMHNSWNG
Plc-N  PDGQRVWHQK GSKGEILPYH FDTSTTS... AQRVDGTPHT WPDAQQAWNE
Plc-H  QDGKPVWYQ. NYKYEFSPYH WDTKVTS... AQWVSSQNHE WSAFHAIWNQ

       201                                               250
HL-A   GANDNWLPAQ AT.TRAGPYV PLTMGYYTRQ DIPIHYLLAD TFTICDGYHC
HL-B   GANDNWLPAQ VPFSPLQGNV PVTMGFYTRR DLPIHYLLAD TFTVCDGYFC
Plc-N  GRMDKWLPAK TERS...... ...LGYYKEQ DIAFQFAMAN AFTICDAYHC
Plc-H  GRNDKWMAVQ YPEA...... ...MGYFKRG DIPYYYALAD AFTLCEAYHQ

       251                                               300
HL-A   SLLTGTLPNR LYWLSANIDP AGTDGGPQLV .....EPGFL PLQQFSWRIM
HL-B   SLLGGTTPNR LYWMSAWIDP DGTDGGPVLI .....EPNIQ PLQHYSWRIM
Plc-N  SFQGGTNPNR LFLWTGTNDP LGQHGGPVTT NDHDSNGPVE QGYT..WTTY
Plc-H  SMMGPTNPNR LYHMSGRAAP SGD.GKDVHI GNDMGDGTIG ASGTVDWTTY

       301                                               350
HL-A   PENLEDAGVS WKVYQNKGLG RF........ ........IN TPISNNGLVQ
HL-B   PENLEDAGVS WKVYQNKLLG AL........ ........NN TVVGYNGLVN
Plc-N  PERLQAAGIT WRVYQDMA.. .......... .......... DNFSDNPLIG
Plc-H  PERLSAAGVD WRVYQEGGYR SSSLWYLYVD AYWKYRLQEQ NNYDGNALAW

       351                                               400
HL-A   AFRQ..AADP RSNLARYGIA PTYPGDFAAD VRANRLPKVS WLVPNILQSE
HL-B   DFKQ..AAD. .......... .......... .......... ..........
Plc-N  .FRQYRAAAP DSPLIVNGLS TWKLDALKRD VLANSLPQVS WIVAPAKYSE
Plc-H  .FRNFKNAPR DSDLWQRAML ARGVDQLRKD VQENTLPQVS WIVAPYCYCE

       401                                               450
HL-A   HPAL.PVALG AVSMVTALRI LLSNPAVWEK TALIVSYDEN GGFFDHVTPP
HL-B   .......... .......... .......... .......... ..........
Plc-N  HPGPSSPIWG AEYTSWVLDA LTANPEVWSK TALLVMFDEN DGFFDHVAPP
Plc-H  HP.WWGPSFG EYYVTRVLEA LTSNPEVWAR TVFILNYDEG DGFYDHASAP

       451                                               500
HL-A   TAPPGTPGEF VTVPNIDAVP GSGGIRG.... ...PLGLGF RVPCIVISPY
HL-B   .......... .......... .......... .......... ..........
Plc-N  AAPSLNKDGT LRGKTTADAT LEWHTKGDIR YRNQPYGLGA RVPMYVISPW
Plc-H  VPPWKDGVGL STVSTAGEIE VSS....... ..GLPIGLGH RVPLIAISPW

       501                                               550
HL-A   SRGPLMVSDT FDHTSQLKLI RARFGVPVPN MTAWRDGVVG DMTSAFNFAT
HL-B   .......... .......... .......... .......... ..........
Plc-N  SKGGWVNSQV FDHTSVIRFL EQRFGVMEPN ISPWRRAVCG DLTSAFNFAN
Plc-H  SKGGKVSAEV FDHTSVLRFL ERRFGLVEEN ISPWRRAVCG DLTSLFDFQG

       551                                               600
HL-A   PPNSTRPNL SHPLLGALPKL PQCIPNVVLG TTDGALPSIP YRVPYPQVMP
HL-B   .......... .......... .......... .......... ..........
Plc-N  PNNE...... ..PF.PELPDT SQADAIVAS. ...QIKLPKP K..PPAVAAM
Plc-H  AGDT...... ..QVAPDLTNV PQSDARKEDA YWQQFYRPSP KYWSYEPKSL

       601                                               650
HL-A   TQETTPVRGT PSGLCS*... .......... .......... ..........
HL-B   .......... .......... .......... .......... ..........
Plc-N  PKQEMGIRPA RALPYELGVH ARYRSGGDAL SLTFANTG.. .......KAG
Plc-H  PGQEKGQRPT LAVPYQLHAT LALDIAAGKL RLTLGNDGMS LPGNPQDTAA

       651                                               700
HL-A   .......... .......... .......... .......... ..........
HL-B   .......... .......... .......... .......... ..........
Plc-N  AVFQVFDLLD SENPPKRYTV GARKRLHDSF QG........ .....DASGD
Plc-H  AVFQV..QPR EVGNPRFYTV TSYPVVQESG EELGRTLNDE LDDLLDANGR

       701                                               750
HL-A   .......... .......... .......... .......... ..........
HL-B   .......... .......... .......... .......... ..........
Plc-N  YHLEVHGPNG FLRVFRGNLR RDLADGKAPL PEVRIDYEPL FGNLRVQLIN
Plc-H  YAFEVHGPNG FFREFHGNLH ..LAAQMAR. PEVSVTYQRN .GNLQLNIRN

       751                                               800
HL-A   .......... .......... .......... .......... ..........
HL-B   .......... .......... .......... .......... ..........
Plc-N  RGRHPVKLTV KDN..VYRQG ERRTVNVPPG QRREVRYSLR SSGNWYDFSV
Plc-H  LGRLPCRRDG DAEPGLYPGG SRRYELEPNQ AISEVWL.LR SSQGWYDLSV

       801                                   836
HL-A   .......... .......... .......... .......
HL-B   .......... .......... .......... .......
Plc-N  SAQGADS.FL RRFSGRMEDG RSGFSDPGMG LGTLTF*
Plc-H  TASNTEANYL RRLAGHVETG KPSRSDPLLD IAAT*..
```

FIG. 2. Comparison of the amino acid sequences of the polypeptides HL-A and HL-B of *M. tuberculosis* with the sequences of the two PLCs, i.e., Plc-N and Plc-H, of *P. aeruginosa*. The protein alignment was done by use of the PILEUP program. Residues are indicated in a single-letter code. Highlighted amino acid residues are identical or conservatively similar between the proteins, and they were grouped as follows: AST, DEQ, ILMV, RK, and FYW.

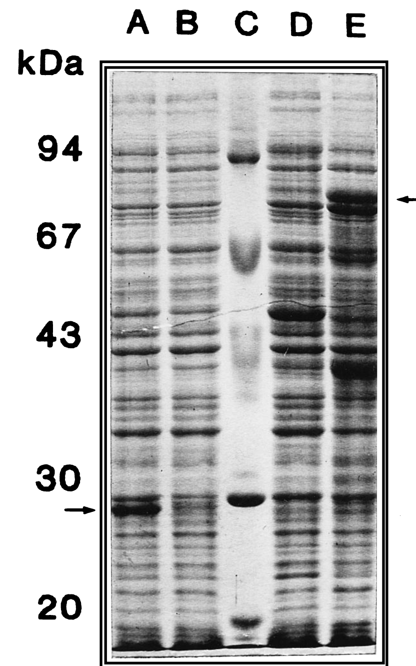

FIG. 3. Expression of the recombinant protein in overnight cultures of *E. coli* transformed with parental and recombinant pGEX-5T. Lanes: A, Induced pGEX-5T; B, noninduced pGEX-5T; C, molecular standards; D, noninduced recombinant pGEX-5T; E, induced recombinant pGEX-5T. The 27-kDa glutathione *S*-transferase and the 83-kDa recombinant protein are indicated by arrows.

from *P. aeruginosa*, the hemolysin inducers do not reveal any homology with toxins in the data banks.

PLC activity has never been demonstrated in mycobacteria, in spite of the role the enzyme appears to play in the virulence mechanisms of other pathogens (6, 16, 22, 28, 36, 37). Wheeler and Ratledge (40) reported phospholipase A1, A2, and lysophospholipase activities of *Mycobacterium leprae*, an organism with a limited capacity to synthesize its own fatty acids. Be-

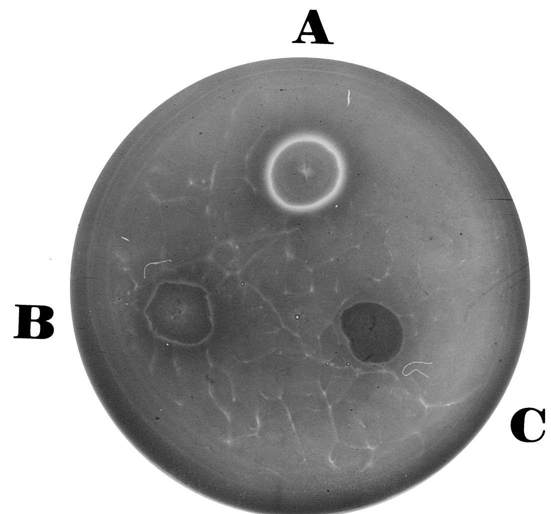

FIG. 4. Hemolytic activity of the recombinant pGEX-5T clone. Recombinant and nonrecombinant clones were plated over blood-agar and incubated for 24 h at 37°C. (A) Induced recombinant pGEX-5T; (B) noninduced recombinant pGEX-5T; (C) induced pGEX-5T.

cause these phospholipases were not expressed at the surface of the bacilli and hydrolyze phospholipids to release fatty acids, they appear to be involved in nutrition rather than in host cell damage.

PLCs in *P. aeruginosa* are said to be responsible for damage to the surfactant of the lungs, allowing bacilli to colonize deeper tissues in this organ (24). PLC also acts cooperatively with alkaline phosphatase as a mechanism to obtain P_i from phospholipids in other microorganisms (7). Although mycobacterial phosphate metabolism is not completely understood, the existence of an *M. tuberculosis* protein induced under phosphate starvation and homologous to the PhoS protein of *E. coli* has been suggested (2). PLCs in *P. aeruginosa* are under control of PhoB and are induced under low phosphate levels in vitro (1). Since we identified a sequence that resembles a Pho-box (32) upstream of the major ORF, it is tempting to conclude that it may represent the same regulatory system in *M. tuberculosis* as it does in *P. aeruginosa.*

Two PLCs appear to play a significant role in listeriosis, contributing to the escape of listeriae from the lysosome, allowing the spread of listeriae from cell to cell (38). *M. tuberculosis* but not BCG was shown by McDonough and collaborators (23) to escape from the phagolysosome several hours after infection. *M. tuberculosis* enzymes similar to those described here could play a role in this phenomenon (23). The escape of *M. tuberculosis* from the phagolysosome has been a matter of controversy. For a long time, it has been considered that pathogens of *Mycobacterium* species and *Salmonella typhimurium* replicated inside the endosomal compartment and did not enter the cytoplasm. However, the identification of class I-restricted CD8 cells with reactivity for these intracellular bacteria (15, 12, 29) and the fact that protection from tuberculosis is considered to be CD8 mediated and class I dependent (15) raised the question of how such antigens are introduced into the class I pathway. Although an alternative pathway for the presentation of class I-restricted antigens has been recently suggested to explain the antigen processing of pathogens that live inside the vacuole (27), the explanation for presentation of class I-restricted antigens in tuberculosis could be that the bacilli escape from the vacuole and/or that, during the long-lasting persistence of *Mycobacterium* infection, the vacuole becomes leaky and allows secreted molecules and/or low-molecular-weight metabolites to enter the class I pathway (18). The production of PLCs by pathogenic *Mycobacterium* spp. is a plausible explanation in either case.

Finally, there are molecules in both bacterial and mammalian cells that could be the natural substrates for these enzymes. Xu et al. have recently demonstrated that lipoarabinomannan is an important component of the small vesicles that bud out from phagosomes infected with *M. tuberculosis* (41). Hunter and Brennan have presented evidence for the presence of a phosphatidylinositol anchor of lipoarabinomannan and lipomannan on the surface of *M. tuberculosis* (19). These molecules have been implicated as major immunogens in tuberculosis infection and are good candidates to be substrates of the enzymes reported here. On the other hand, since bacterial and mammalian PLCs have some similarity (10), one could speculate that during infection, PLCs from *M. tuberculosis* compete with mammalian PLCs for their substrates, e.g., arachidonic acid, interfering with the normal generation of diacyl-glycerol and inositol triphosphate, important metabolites in cell activation, rendering the cells unable to control the intracellular growth of this pathogen.

In conclusion, an *M. tuberculosis* DNA fragment encoding putative polypeptides similar to PLC has been identified. The presence of the native protein in sonic extracts and/or culture filtrates of *M. tuberculosis* as well as full PLC activity are still to be demonstrated. Further characterization of these putative proteins could help in the understanding of the demonstrated escape from the phagolysosome and the cytolytic activity of this pathogen, could help in the elucidation of the phosphate metabolism of mycobacteria, and could also contribute to the characterization of what could turn out to be an important virulence factor in *M. tuberculosis*. In the long term, these studies may contribute to the rational design of better candidate vaccines or enzyme inhibitors with potential therapeutic use against tuberculosis.

ACKNOWLEDGMENTS

We thank Julieta Flórez and Carlos Yesid Soto for technical support.

This work was supported by the Colombian Ministry of Health, the German Leprosy Relief Association, the Instituto Colombiano para el Desarrollo de la Ciencia y la Tecnología, Francisco José de Caldas (COLCIENCIAS) grant N.011-93, and the Heiser Program for Research in Leprosy and Tuberculosis.

REFERENCES

1. **Anba, J., M. Bidaud, M. L. Vasil, and A. Lazdunski.** 1990. Nucleotide sequence of the *Pseudomonas aeruginosa phoB* gene, the regulatory gene for the phosphate regulon. J. Bacteriol. **172:**4685–4689.
2. **Andersen, A. B., L. Ljungqvist, and M. Olsen.** 1990. Evidence that protein antigen b of *Mycobacterium tuberculosis* is involved in phosphate metabolism. J. Gen. Microbiol. **136:**477–480.
3. **Armstrong, J. A., and P. D. Hart.** 1971. Response of cultural macrophages to *Mycobacterium tuberculosis*, with observations on fusion of lysosomes and phagosomes. J. Exp. Med. **134:**713–740.
4. **Arruda, S., G. Bomfim, R. Knights, T. Huima-Byron, and L. W. Riley.** 1993. Cloning of an *M. tuberculosis* DNA fragment associated with entry and survival inside cells. Science **261:**1454–1457.
5. **Bannan, J. D., M. J. Moran, J. I. MacInnes, G. A. Soltes, and R. L. Friedman.** 1993. Cloning and characterization of *btr*, a *Bordetella pertussis* gene encoding an FNR-like transcriptional regulator. J. Bacteriol. **175:**7228–7235.
6. **Berka, R. M., G. L. Gray, and M. L. Vasil.** 1981. Studies of phospholipase C (heat-labile hemolysin) in *Pseudomonas aeruginosa*. Infect. Immun. **34:**1071–1074.
7. **Berka, R. M., and M. L. Vasil.** 1982. Phospholipase C (heat-labile hemolysin) of *Pseudomonas aeruginosa*: purification and preliminary characterization. J. Bacteriol. **152:**239–245.
8. **Berthold, H., B. Frorath, M. Scanarini, C. C. Abney, B. Ernst, and W. Northemann.** 1992. Plasmid pGEX-5T: an alternative system for expression and purification of recombinant proteins. Biotechnol. Lett. **14:**245–250.
9. **Bielecki J., P. Youngman, P. Connelly, and D. A. Portnoy.** 1990. *Bacillus subtilis* expressing a haemolysin gene from *Listeria monocytogenes* can grow in mammalian cells. Nature (London) **345:**175–176.
10. **Clark, M. A., R. G. L. Schorr, and J. S. Bomalaski.** 1986. Antibodies prepared to *Bacillus cereus* phospholipase C crossreact with a phosphatidylcholine preferring phospholipase C in mammalian cells. Biochem. Biophys. Res. Commun. **140:**114–119.
11. **Dale, J. W., and A. Patki.** 1990. Mycobacterial gene expression and regulation, p. 173–198. *In* J. McFadden (ed.), Molecular biology of mycobacteria. Surrey University Press, Academic Press Ltd., London.
12. **DeLibero, G., I. Flesch, and S. H. E. Kaufmann.** 1988. Mycobacteria reactive Lyt2+T cell lines. Eur. J. Immunol. **18:**59–66.
13. **Fields, P. I., E. A. Groisman, and F. Heffron.** 1989. A Salmonella locus that controls resistance to microbicidal proteins from phagocytic cells. Science **243:**1059–1062.
14. **Fine, P. E. M.** 1989. The BCG story: lessons from the past and implications for the future. Rev. Infect. Dis. **11**(Suppl. 2):S353–S359.
15. **Flynn, J. L., W. R. Weiss, K. A. Norris, H. S. Seifert, S. Kummar, and M. So.** 1990. Generation of a cytotoxic T-lymphocyte response using a *Salmonella* antigen-delivery system. Mol. Microbiol. **4:**2111–2118.
16. **Gilmore, M. S., A. L. Cruz-Rodz, M. Leimeister-Wächter, J. Kreft, and W. Goebel.** 1989. A *Bacillus cereus* cytolytic determinant, cereolysin AB, which comprises the phospholipase C and sphingomyelinase genes: nucleotide sequence and genetic linkage. J. Bacteriol. **171:**744–753.
17. **Gordon, A. H., P. D'Arcy Hart, and M. R. Young.** 1980. Ammonia inhibits phagosome-lysosome fusion in macrophages. Nature (London) **286:**79–80.
18. **Hart, P. D., J. A. Armstrong, C. A. Brown, and P. Draper.** 1972. Ultrastructural study of the behavior of macrophages toward parasitic mycobacteria. Infect. Immun. **5:**803–807.
19. **Hunter, S. W., and P. J. Brennan.** 1990. Evidence for the presence of a phosphatidylinositol anchor on the lipoarabinomannan and lipomannan of

Mycobacterium tuberculosis. J. Biol. Chem. **265:**9272–9279.
20. **King, C. H., S. Mundayoor, J. T. Crawford, and T. M. Shinnick.** Expression of contact-dependent cytolytic activity by *Mycobacterium tuberculosis* and isolation of the genomic locus that encodes the activity. Infect. Immun. **61:**2708–2712.
21. **Leão, S. C.** 1993. Tuberculosis: new strategies for the development of diagnostic tests and vaccines. Braz. J. Med. Biol. Res. **26:**827–833.
22. **Logan, A. J., E. D. Williamson, R. W. Titball, D. A. Percival, A. D. Shuttleworth, J. W. Conlan, and D. C. Kelly.** 1991. Epitope mapping of the alpha-toxin of *Clostridium perfringens*. Infect. Immun. **59:**4338–4342.
23. **McDonough, K. A., Y. Kress, and B. R. Bloom.** 1993. Pathogenesis of tuberculosis: interaction of *Mycobacterium tuberculosis* with macrophages. Infect. Immun. **61:**2763–2773.
24. **Ostroff, R. M., A. I. Vasil, and M. L. Vasil.** 1990. Molecular comparison of a nonhemolytic and a hemolytic phospholipase C from *Pseudomonas aeruginosa*. J. Bacteriol. **172:**5915–5923.
25. **Parra, C. A., L. P. Londoño, P. del Portillo, and M. E. Patarroyo.** 1991. Isolation, characterization, and molecular cloning of a specific *Mycobacterium tuberculosis* antigen gene: identification of a species-specific sequence. Infect. Immun. **59:**3411–3417.
26. **Pearson, W. R., and D. J. Lipman.** 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **88:**2444–2448.
27. **Pfeifer, J. D., M. J. Wick, R. L. Roberts, K. Findlay, S. J. Normark, and C. V. Harding.** 1993. Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. Nature (London) **361:**359–362.
28. **Pritchard, A. E., and M. L. Vasil.** 1986. Nucleotide sequence and expression of a phosphate-regulated gene encoding a secreted hemolysin of *Pseudomonas aeruginosa*. J. Bacteriol. **167:**291–298.
29. **Rees, A. D. M., A. Scoging, A. Mehlert, D. B. Young, and J. Ivanyi.** 1988. Specificity of proliferative response of human CD8 clones to mycobacterial antigens. Eur. J. Immunol. **18:**1881–1887.
30. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
31. **Sibley, L. D., J. L. Krahenbuhl, G. M. W. Adams, and E. Weidner.** 1986. Toxoplasma modifies phagosomes by secretion of a vesicular network rich in surface proteins. J. Cell Biol. **103:**867–874.
32. **Smith, N. W., and J. W. Payne.** 1992. Expression of periplasmic binding proteins for peptide transport is subject to negative regulation by phosphate limitation in *Escherichia coli*. FEMS Microbiol. Rev. **100:**183–190.
33. **Snider, D. E., and W. L. Roper.** 1992. The new tuberculosis. N. Engl. J. Med. **326:**703–705.
34. **Soltes, G. A., and J. I. MacInnes.** 1994. Regulation of gene expression by the HlyX protein of *Actinobacillus pleuropneumoniae.* Microbiology **140:**839–845.
35. **Sturgyll-Koszycki, S., P. H. Schlesinger, P. Chakraborty, P. L. Haddix, H. L. Collins, A. K. Fok, R. D. Allen, S. L. Gluck, J. Heuser, and D. G. Russel.** 1994. Lack of acidification in *Mycobacterium* produced by exclusion of the vesicular proton-ATPase. Science **263:**678–681.
36. **Titball, R. W., S. E. C. Hunter, K. L. Martin, B. C. Morris, A. D. Shuttleworth, T. Rubidge, D. W. Anderson, and D. C. Kelly.** 1989. Molecular cloning and nucleotide sequence of the alpha-toxin (phospholipase C) of *Clostridium perfringens*. Infect. Immun. **57:**367–376.
37. **Vasil, M. L., R. M. Berka, G. L. Gray, and H. Nakai.** 1982. Cloning of a phosphate-regulated hemolysin gene (phospholipase C) from *Pseudomonas aeruginosa*. J. Bacteriol. **152:**431–440.
38. **Vazquez-Boland, J.-A., C. Kocks, S. Dramsi, H. Ohayon, C. Geoffroy, J. Mengaud, and P. Cossart.** 1992. Nucleotide sequence of the lecithinase operon of *Listeria monocytogenes* and possible role of lecithinase in cell-to-cell spread. Infect. Immun. **60:**219–230.
39. **Watson, M. E. E.** 1984. Compilation of published signal sequences. Nucleic Acids Res. **12:**5145–5164.
40. **Wheeler, P. R., and C. Ratledge.** 1991. Phospholipase activity of *Mycobacterium leprae* harvested from experimentally infected armadillo tissue. Infect. Immun. **59:**2781–2789.
41. **Xu, S., A. Cooper, S. Sturgyll-Koszycki, T. van Heyningen, D. E. Chatterjee, I. Orme, and D. G. Russell.** 1994. Intracellular trafficking in *Mycobacterium tuberculosis* and *Mycobacterium avium*-infected macrophages. J. Immunol. **153:**2568–2578.

Editor: B. I. Eisenstein