

Phospholipases C are involved in the virulence of *Mycobacterium tuberculosis*

Catherine Raynaud,^{1*†} Christophe Guilhot,^{1‡}
Jean Rauzier,¹ Yann Bordat,¹ Vladimir Pelicic,²
Riccardo Manganeli,³ Issar Smith,⁴ Brigitte Gicquel¹
and Mary Jackson¹

¹Unité de Génétique Mycobactérienne, Institut Pasteur,
75724 Paris, France.

²U411 INSERM, Faculté Necker, 750015 Paris, France.

³Dept of Histology, Microbiology and Medical
Biotechnologies, University of Padova, 35121 Padova,
Italy.

⁴The Public Health Research Institute, Newark,
NJ 07103–3535, USA.

Summary

Phospholipases C play a role in the pathogenesis of several bacteria. *Mycobacterium tuberculosis*, the causative agent of tuberculosis, possesses four genes encoding putative phospholipases C, *plcA*, *plcB*, *plcC* and *plcD*. However, the contribution of these genes to virulence is unknown. We constructed four single mutants of *M. tuberculosis* each inactivated in one of the *plc* genes, a triple *plcABC* mutant and a quadruple *plcABCD* mutant. The mutants all exhibited a lower phospholipase C activity than the wild-type parent strain, demonstrating that the four *plc* genes encode a functional phospholipase C in *M. tuberculosis*. Functional complementation of the Δ *plcABC* triple mutant with the individual *plcA*, *plcB* and *plcC* genes restored in each case about 20% of the total Plc activity detected in the parental strain, suggesting that the three enzymes contribute equally to the overall Plc activity of *M. tuberculosis*. RT-PCR analysis of the *plc* genes transcripts showed that the expression of these genes is strongly upregulated during the first 24 h of macrophage infection. Moreover, the growth kinetics of the triple and quadruple mutants in a mouse model of infection revealed that both mutants are attenuated in the late phase of the infection emphasizing the importance of phospholipases C in the virulence of the tubercle bacillus.

Introduction

Phospholipases are important virulence factors in an increasing number of intra- and extracellular bacterial pathogens including *Clostridium perfringens*, *Corynebacterium pseudotuberculosis*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Listeria monocytogenes* (McNamara *et al.*, 1994) (for reviews, see Titball, 1993; Songer, 1997). Phospholipases can be divided into four groups depending on the position of the bond they hydrolyse on the phospholipid substrate: phospholipases A1, A2, C and D. Phospholipases C (Plc) appear to be the most important playing a significant role in bacterial pathogenesis (Songer, 1997). For example, in the Gram-positive intracellular pathogen *L. monocytogenes*, the inactivation of the *plcA* gene encoding a phosphatidylinositol-specific Plc, prevents replication within mouse peritoneal macrophages, cell-to-cell spread and propagation in host tissues (Camilli *et al.*, 1991). The α toxin (CPA) from *C. perfringens* is the most toxic Plc characterized to date. It has haemolytic, lethal, dermonecrotic, vascular permeabilizing and platelet-aggregating properties (Titball, 1993). The haemolytic phospholipase C of *P. aeruginosa* (PlcHR) suppresses the bacterium-induced neutrophil respiratory burst by interfering with a protein kinase C-specific signalling pathway (Terada *et al.*, 1999).

Tuberculosis remains the leading cause of death due to a single infectious organism in the world. Despite the considerable amount of work devoted to deciphering the molecular basis of *Mycobacterium tuberculosis* pathogenicity, little is known about the mechanisms enabling this pathogen to resist destruction by the host and to multiply inside mononuclear phagocytic cells. Owing to their role in the pathogenesis of many bacterial pathogens, phospholipases have been studied in mycobacteria. Phospholipase C (Plc) and phospholipase D (Pld) activities have been described in several mycobacterial species. However, although Pld activity has been detected in both virulent and saprophytic species, Plc and sphingomyelinase activities seem to be restricted to pathogenic *Mycobacterium* subsp. (Johansen *et al.*, 1996). For example, cell extracts of *M. tuberculosis* and *Mycobacterium ulcerans*, the causative agents of tuberculosis and Buruli ulcer, respectively, contain both Plc and Pld activities, whereas only Pld activity has been reported in cell extracts of the non-pathogenic *Mycobacterium smegmatis* (Johansen *et al.*, 1996; Gomez *et al.*, 2000). Despite this striking correlation, the role of phospholi-

Accepted 8 April, 2002. *For correspondence. E-mail cathraynaud@yahoo.fr; Tel. (+44) 2089593666; Fax (+44) 2089138528. Present addresses: [†]Division of Mycobacterial Research, The Ridgeway, Mill Hill, London NW7 1AA, UK. [‡]IPBS-CNRS, 205 Route de Narbonne, 31077 Toulouse Cedex, France.

pases C in the pathogenicity of *M. tuberculosis* had not yet been investigated.

The genome sequences of *M. tuberculosis* H37Rv (Cole *et al.*, 1998) and CDC1551 (<http://www.tigr.org>) revealed the presence of four highly related genes encoding putative phospholipases, namely *plcA*, *plcB*, *plcC* and *plcD*. The *plcA*, *plcB* and *plcC* genes are clustered on the chromosome, whereas *plcD* is located in a different region. The enzymes encoded by these genes share 30% to 40% of overall amino acid identity with the PlcH and PlcN phospholipases C of *P. aeruginosa* and about 70% amino acid identity between them. Expression of the *M. tuberculosis plcA* and *plcB* genes in *M. smegmatis*

conferred upon this bacterium both sphingomyelinase and phospholipase C activities (Johansen *et al.*, 1996). In addition, cell extracts from an *Escherichia coli* strain producing a recombinant PlcA protein exhibited β -haemolytic activity (Leão *et al.*, 1995). The *plcC* and *plcD* genes whose existence was revealed upon sequencing of the *M. tuberculosis* genome have not yet been functionally characterized. The *plcD* gene is truncated and interrupted by a copy of the IS6110 insertion sequence in the laboratory strain *M. tuberculosis* H37Rv, but not in the clinical isolate CDC1551. Comparison of the structure of the *plcD* region in seven clinical isolates of *M. tuberculosis* revealed that three of the isolates carried the truncated version of the

Table 1. Primers used in this study.

Name	Sequence
Screening of the <i>M. tuberculosis</i> Mt103 transposon mutant library for <i>plcA</i> , <i>plcB</i> and <i>plcC</i> mutants	
1	5'-GTGACTTAAGCGCCGAAGCCGGCCG-3'
2	5'-CCCTGCCGGTAGCGCTTGCGCGGT-3'
3	5'-CGGCTCTACTGGATGAGCGCCTGGA-3'
4	5'-AGGCGCGGACCCGGTCGGTGGCCAA-3'
5	5'-TCCGGACGGTGTACGCGTCGCCAC-3'
OP	5'-TTTGAGCTCTACACCGTCAAGTGCGAAGAGC-3'
Construction of the $\Delta plcABC$ mutant	
7	5'-GGGAATTCGGACGGTGTACGCGTCGCCA-3'
8	5'-GGGCTCGAGCAAGCGCTACCGCAGGGCGG-3'
9	5'-GGGCTCGAGCGGGTTCGACACTCCGACGCC-3'
10	5'-GGGAATTCGCCGGCCCGGAGAAATGCGG-3'
Construction of the <i>plcD</i> and <i>plcABCD</i> mutants	
11	5'-ATAAGAATGCGGCCGTGAAATGCGGCTGGAGGTTTCG-3'
12	5'-GGGAAGCTTCCAATACCGAGCAAAAGTAGCGG-3'
13	5'-CCCAAGCTTGGGTCCCACCTGCCCAACC-3'
14	5'-GGACTAGTTTAGCACGGACCGCTCGGAAT-3'
Amplification of <i>plcA</i> , <i>plcB</i> , <i>plcC</i> and <i>plcD</i> with Turbo pfu DNA polymerase	
15	5'-GAAGATCTATGTCACGTCGAGAGTTTTTGACAA-3'
16	5'-CCCTCTAGAGGTACCGCACAGCCCGCTGGCAG-3'
17	5'-GAAGATCTATGACCCGCGACAATTTTTTG-3'
18	5'-CCCTCTAGAGGTACACAGAGACCGCTGGGAA-3'
19	5'-GAAGATCTATGTCACGCGGAGCATTCTG-3'
20	5'-CCCTCTAGAGGTACCGATGCCCTGGGAATC-3'
21	5'-CGGGATCCGTGAGCCAAAGCCACATCGG-3'
22	5'-CCGGTACCACCGCTCGGAATACCACGGGTA-3'
1. RT-PCR	
23	a,5'-CGCAGGCGACCACCCGCG-3'; b,5'-CGGCAGGAAGCCCGGCTCT-3'
24	a,5'-TGCTCGTGCTGGGCGGGA-3'; b,5'-CGTTGTAGCCGACGACGGTG-3'
25	a,5'-GGCCTATGGTGCCGGTCCC-3'; b,5'-GGTGGGTCCAGCGCCTGCG-3'
26	a,5'-CGGGGCGGATCGGTTTGGG-3'; b,5'-TTAGCACGGACCGCTCGGAAT-3'
2. RT-PCR	
<i>sigA-R</i>	5'-CTGACATGGGGGCCGCTACCGTTG-3'
<i>plcA-R</i>	5'-TGACCGTTCTCCTGC-3'
<i>plcB-R</i>	5'-CGCGGGTGTGAGAAAG-3'
<i>plcC-R</i>	5'-GACCGGCACCATAGGC-3'
<i>plcD-R</i>	5'-TAGCTTGGTGCCACGC-3'
Quantitative PCR	
<i>sigA</i>	a,5'-GGCCAGCCGCGCACCCCTTAC-3'; b,5'-GTCCAGCTAGTCGCGCAGGACC-3'
<i>plcA</i>	a,5'-CCGTTCTCCTGCATC-3'; b,5'-TGGACTGGGCTGCAC-3'
<i>plcB</i>	a,5'-GACCCGCGACAATTT-3'; b,5'-CCCCTCCGTAGGCTT-3'
<i>plcC</i>	a,5'-TGTCACGCCGAGCATT-3'; b,5'-GGTGCGGCCAGTCC-3'
<i>plcD</i>	a,5'-GGCGTCAGCTGGAAGG-3'; b,5'-TAGCTTGGTGCCACGC-3'
<i>sigA</i>	Beacon: 5'-CCTCGCGTCAAGTTGCGCCATCCGAGCGAGG-3'

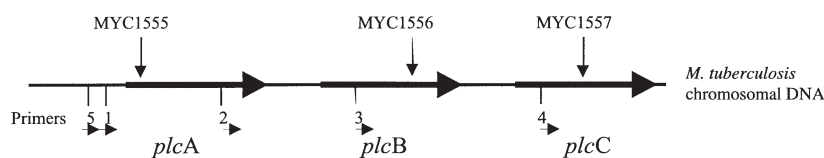


Fig. 1. Identification of *plcA*, *plcB* and *plcC* insertional mutants within an ordered *Mycobacterium tuberculosis* transposon mutant library. The following primer pairs were used to perform the PCR reactions: OP/1, OP/2, OP/3, OP/4 and OP/5 (Table 1). The sites at which the transposon was inserted in the MYC1555 (*plcA*), MYC1556 (*plcB*) and MYC1557 (*plcC*) mutants are represented by black arrows.

plcD gene (Gordon *et al.*, 1999). The *plc* gene polymorphism is not restricted to *plcD* as shown by the fact that the *M. tuberculosis* genomic region encompassing the *plcABC* locus (region of deletion RD5) is absent from *Mycobacterium bovis*, *M. bovis* BCG and *Mycobacterium microti* (Gordon *et al.*, 1999).

As phospholipases C may contribute to the pathogenesis of several important pathogens, we investigated the role of the *plc* genes in the virulence of *M. tuberculosis* by constructing and analysing *plc* mutant strains.

Results

Construction of *plcA*, *plcB*, *plcC*, *plcD*, *plcABC* and *plcABCD* mutants of *M. tuberculosis*

We used the clinical isolate of *M. tuberculosis* Mt103 for all gene inactivation experiments. Because of the polymorphism affecting the *plcABC* and *plcD* genomic regions in the different species of the *M. tuberculosis* complex, we first confirmed that the *plcA*, *plcB*, *plcC* and *plcD* genes were all present in this isolate. The four full-length genes were amplified by polymerase chain reaction (PCR) by use of the following primer pairs: 15/16 (*plcA*), 17/18 (*plcB*), 19/20 (*plcC*) and 21/22 (*plcD*) (Table 1). In each case, an amplification product of the expected size (approximately 1.5 kb) was obtained and sequenced. For *plcA*, *plcB* and *plcC*, the sequence was 100% identical to that of *M. tuberculosis* H37Rv. In contrast to the situation in H37Rv, a non-truncated and undisrupted copy of the *plcD* gene was present in Mt103. Its sequence was 100% identical to that of *M. bovis* BCG and CDC1551. Therefore, strain Mt103 contains four full-length phospholipase C genes.

Two independent approaches were used to isolate insertional mutants of Mt103 deficient in the expression of the *plc* genes. First, we used PCR to screen 6912 *M. tuberculosis* clones from a transposon mutant library for the presence of transposon insertions within the *plc* genes as described by Jackson and colleagues (Jackson *et al.*, 1999). The sequences of the primers used (1, 2, 3, 4, 5 and OP) are shown in Table 1. Primers 1–5 are 25mers designed to anneal different regions of the *plcABC* locus (Fig. 1). Primer OP is specific for the inverted repeats (IR) of IS1096 and is directed outward the transposon. Combinations of primers (1/OP, 2/OP, 3/OP, 4/OP and 5/OP)

were used to amplify the DNA regions between the respective primers and putative transposon insertion sites within the *plc* genes. This method enabled us to isolate mutants harbouring transposon insertions in the *plcA*, *plcB* and *plcC* genes (Fig. 1). The presence of a transposon in each of these genes was further confirmed by Southern blotting (data not shown). The sequence of the PCR amplification products determined that the transposon was inserted 18 bp downstream from the *plcA* start codon in the *plcA* mutant, 1168 bp downstream from the *plcB* start codon in the *plcB* mutant and 813 bp downstream from the *plcC* start codon in the *plcC* mutant. The *plcA*, *plcB* and *plcC* mutants were named MYC1555, MYC1556 and MYC1557 respectively (Table 2).

A *plcABC* triple mutant was constructed by allelic exchange using the *Ts/sacB* vectors described by Pelicic and collaborators (Pelicic *et al.*, 1997). An inactive copy of the *plcABC* cluster was generated by PCR. The PCR product contained the *plcABC* genomic region lacking a 2.6 kb internal DNA fragment encompassing the last 600 bp of the *plcA* gene, the entire *plcB* gene and the first 200 bp of the *plcC* gene (Fig. 2A). A kanamycin resistance cassette was cloned into the deleted *plcABC* locus, yielding the disrupted allele *plcABC::km*. This allele and the *xylE* gene were then cloned into pPR27, a plasmid carrying a temperature-sensitive origin of replication and the *sacB* counter-selectable marker (Pelicic *et al.*, 1997). The resulting plasmid, p27PKX, was used to achieve allelic replacement at the *plcABC* locus of *M. tuberculosis* Mt103. When selection procedure was applied, 100% of the Kan^r, Suc^r colonies selected were *plcABC* allelic exchange mutants, as confirmed by Southern blotting (Fig. 2A). One *plcABC* mutant was selected and named MYC1558 (Table 2).

Following the same procedure, a disrupted copy of the *plcD* gene (*plcD::hyg*) was constructed and cloned into the *Ts/sacB* vector pPR23 (Pelicic *et al.*, 1997) yielding plasmid pCR8, the vector used to achieve allelic exchange at the *plcD* locus (Fig. 2B). *M. tuberculosis* Mt103 and MYC1558 were transformed with this plasmid, and allelic exchange mutants were selected using sucrose counter-selection at a non-permissive temperature. Both *plcD* and *plcABCD* mutants were obtained as confirmed by PCR analysis and Southern blotting (Fig. 2B). The *plcD*-deficient mutant was named

Table 2. Plasmids and strains used in this study.

Plasmids	Relevant characteristics	Source or reference
pGEM-T	Amp ^r , cloning vector for PCR products	Promega
pBS (+/-)	Amp ^r , cloning vector	Stratagene
pPR27	Gent ^r , Suc ^r , cloning vector	V. Pelicic <i>et al.</i> (1997)
pPR23	Gent ^r , Suc ^r , cloning vector	V. Pelicic <i>et al.</i> (1997)
pMIP12	Km ^r , <i>E. coli</i> / <i>Mycobacterium</i> shuttle vector	Le Dantec <i>et al.</i> (2001)
pMIP12H	Hyg ^r , <i>E. coli</i> / <i>Mycobacterium</i> shuttle vector	Le Dantec <i>et al.</i> (2001)
pCR1	Km ^r , pMIP12 containing <i>plcA</i> structural gene	This study
pCR2	Km ^r , pMIP12 containing <i>plcB</i> structural gene	This study
pCR3	Km ^r , pMIP12 containing <i>plcC</i> structural gene	This study
pCR5	Hyg ^r , pMIP12H containing <i>plcA</i> structural gene	This study
pCR6	Hyg ^r , pMIP12H containing <i>plcB</i> structural gene	This study
pCR7	Hyg ^r , pMIP12H containing <i>plcC</i> structural gene	This study
pCR8	Hyg ^r , pPR23 containing <i>plcD::hyg</i>	This study
PCR9	Hyg ^r -Km ^r , pIPX59 containing the <i>plcABC</i> genes	This study
p27PKX	Km ^r , pPR27 containing Δ <i>plcABC::Km + xylE</i>	This study
Strains		
mc ² 155	<i>Mycobacterium smegmatis</i>	Snapper <i>et al.</i> (1990)
H37Rv	<i>Mycobacterium tuberculosis</i>	Steenken <i>et al.</i> (1946)
Mt103	<i>Mycobacterium tuberculosis</i> (wild type)	Clinical isolate
MYC1555	Mt103 <i>plcA::Tn5367</i> , Km ^r	This study
MYC1556	Mt103 <i>plcB::Tn5367</i> , Km ^r	This study
MYC1557	Mt103 <i>plcC::Tn5367</i> , Km ^r	This study
MYC1558	Mt103 Δ <i>plcABC</i> , Km ^r	This study
MYC2508	Mt103 <i>plcD::hyg</i> , Hyg ^r	This study
MYC2509	MYC1558 <i>plcD::hyg</i> , Hyg ^r and Km ^r	This study
MYC2510	MYC1558 (pCR9), Hyg ^r and Km ^r	This study
MYC2501	Mt103 (pCR1), Km ^r	This study
MYC2502	Mt103 (pCR2), Km ^r	This study
MYC2503	Mt103 (pCR3), Km ^r	This study
MYC2505	MYC1558 (pCR5), Hyg ^r and Km ^r	This study
MYC2506	MYC1558 (pCR6), Hyg ^r and Km ^r	This study
MYC2507	MYC1558 (pCR7), Hyg ^r and Km ^r	This study
MYC2510	MYC1558 (pCR9), Hyg ^r and Km ^r	This study

MYC2508 and the quadruple *plcABCD* mutant was named MYC2509 (Table 2).

Mt103 and the various mutant strains all exhibited the same colony morphology on plates and the same apparent growth rate in Middlebrook 7H9 medium (Fig. 3A). Likewise, the growth rates were similar when the triple mutant (MYC1558) was complemented with one or all of the *plc* genes (Fig. 3B).

The genetic organization of the *plcABC* cluster suggests that these three genes are co-transcribed. Therefore, we performed reverse transcription (RT)-PCR experiments to investigate whether the transposon insertions within *plcA* and *plcB* had a polar effect on the transcription of the downstream *plc* genes (Fig. 4). In each of the MYC1555, MYC1556 and MYC1557 mutants, transcripts corresponding to the intact *plc* genes were detected. These results suggest that in all three mutants, the non-targeted Plc proteins are produced, although the amounts of each protein may differ from those found in the wild-type Mt103 strain.

Phospholipase C activity of the mutant strains

We used a spectrophotometric assay to monitor the

phospholipase C activity of cell extracts from wild-type *M. tuberculosis* Mt103 and *plc* mutants. Cell extracts consisting of a crude preparation of cytosols, membranes and cell walls were used, rather than whole cells, to enable us to standardize the amount of total proteins used in the assays (500 µg). This assay detects the hydrolysis of a chromogenic derivative of phosphatidylcholine (PC), *p*-nitrophenylphosphorylcholine (pNPPC) (Kurioka and Matsuda, 1976). pNPPC is a specific substrate of phospholipases C that releases upon hydrolysis *p*-nitrophenol that absorbs the light at 410 nm. Measurements were performed in triplicates. The Plc activities of the cell extracts were measured after 1, 6, 14, 18 and 36 h of incubation with pNPPC (Fig. 5A). The Plc activity of the various strains increased for the first 18 h before reaching a plateau. After 18 h, the Plc activity of the single mutants MYC1555, MYC1556, MYC1557 and MYC2508 was significantly reduced (25% decrease for MYC2508 and more than 50% decrease for MYC1555, MYC1556 and MYC1557). Interestingly, MYC1555, MYC1556 and MYC1557 cell extracts had similar Plc activities. As expected, the triple (MYC1558) and quadruple (MYC2509) mutants exhibited the most drastic decreases in Plc activity (70%–80% and 85%–90% respectively)

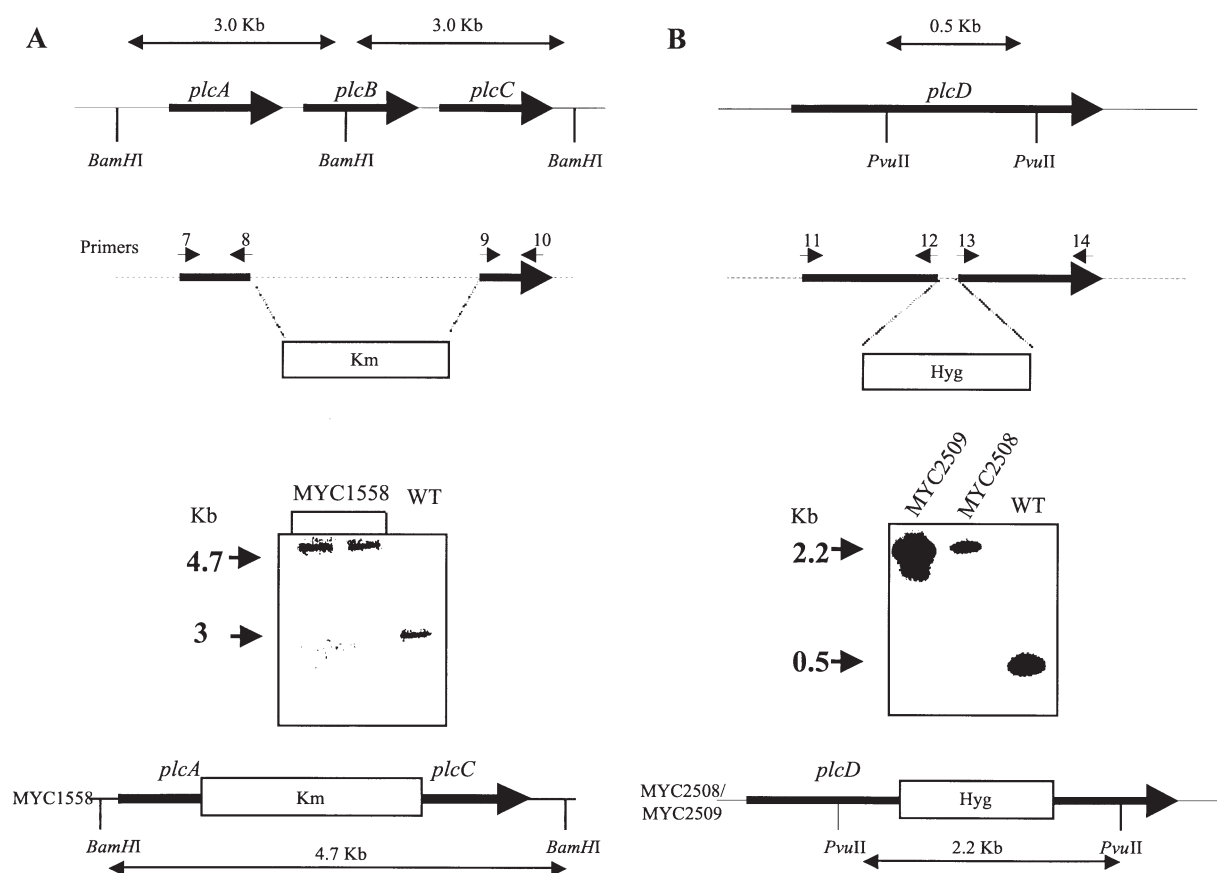


Fig. 2. Construction of *plcD*, *plcABC* and *plcABCD* mutants by allelic replacement.

A. Construction of MYC1558 ($\Delta plcABC$). The *plcABC::Km* allele was used to disrupt the chromosomal *plcABC* locus by allelic replacement. Southern blot of *Bam*HI-cut chromosomal DNA from *M. tuberculosis* Mt103 and two independent clones of MYC1558. The probe used corresponds to the PCR-amplified *plcABC*-deleted locus. Two hybridization signals were detected at about 3 kb for the wild-type strain and one signal was detected at about 4.7 kb for MYC1558, confirming that the *plcABC* locus had been disrupted in the mutant strains.

B. Construction of the MYC2508 (*plcD*) and MYC2509 (*plcABCD*) mutants. Southern blot of *Pvu*II-cut DNA from *M. tuberculosis* Mt103, MYC2508 and MYC2509 using the *plcD* gene (PCR-amplified with primers 21/22) as a probe confirmed the disruption of the *plcD* gene in the mutant strains.

(Fig. 5). Despite the fact that *pNPPC* is considered to be a specific substrate for phospholipases C, it is possible that some phospholipase D or other lipase activities account for the residual activity detected in the cell extracts of the quadruple mutant MYC2509.

To address the functionality of each Plc enzyme in *M. tuberculosis*, we carried out a complementation analysis. Each of the *plcA*, *plcB* or *plcC* genes was introduced individually into the mycobacterial expression vector, pMIP12H, under control of the *pBlaF** promoter (see Table 2). In addition, a 5497 bp blunt-ended *Bpl*-*Mse*I restriction fragment from cosmid MTCY98 carrying the entire *plcABC* cluster and upstream region was inserted into pIPX59, a mycobacterial integrative vector harbouring a kanamycin and a hygromycin resistance gene (Berthet *et al.*, 1998). MYC1558 was transformed with each of the four constructs and phospholipase C activities of the recombinant strains were assayed as described previously. Complementation of the triple mutant with

each of the individual genes *plcA*, *plcB* or *plcC* restored 20% of the Plc activity, whereas complementation with the entire *plcABC* cluster restored full Plc activity (Fig. 5B). The Student's *t*-test confirmed that the Plc activity was significantly higher in each of the complemented strains than in MYC1558 ($p < 0.05$). These experiments demonstrate that all four Plc enzymes are involved in the Plc activity of *M. tuberculosis* and that all four enzymes are functional. Moreover, phosphatidylcholine is not only a potential substrate for PlcA and PlcB as reported earlier (Johansen *et al.*, 1996) but also for PlcC and PlcD.

Subcellular localization of *M. tuberculosis* phospholipases C

Most bacterial phospholipases C are secreted proteins. The *M. tuberculosis* *plc* genes encode proteins with putative signal sequences, suggesting that they are secreted. To investigate the subcellular localization of the phos-

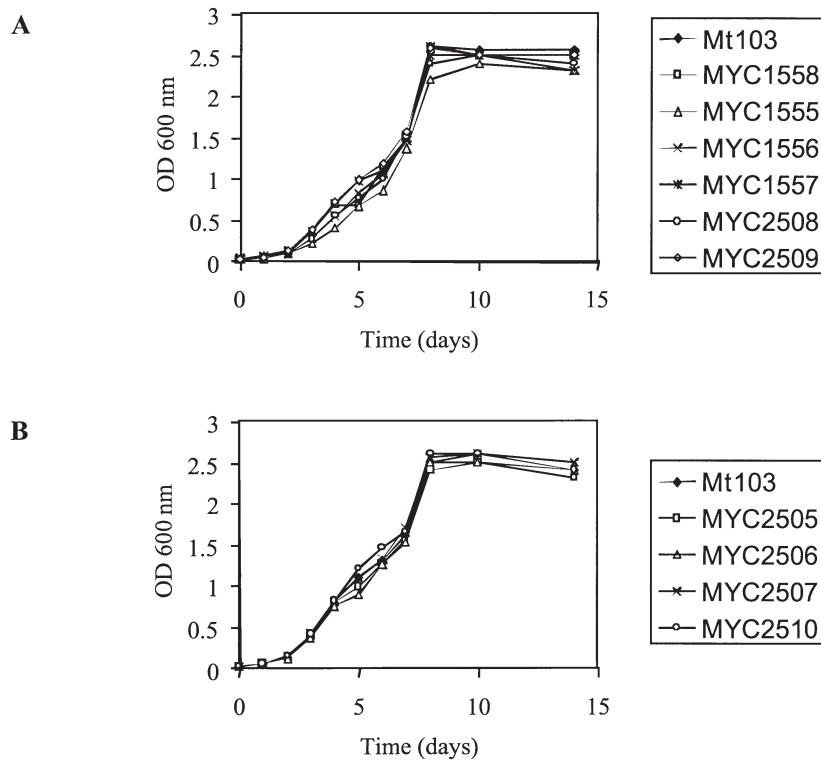


Fig. 3. A. Growth kinetics of *M. tuberculosis* Mt103 and *plc* mutants grown in 7H9 Middlebrook medium at 37°C with agitation. B. Growth kinetics of *M. tuberculosis* Mt103 and the complemented triple mutant strains (see Table 2) grown in 7H9 Middlebrook medium at 37°C with agitation.

pholipases C in *M. tuberculosis*, we checked for the presence of recombinant His-tagged PlcA, PlcB and PlcC proteins in different cell fractions. Each of the three *plc* genes was placed in a *Mycobacterium/E. coli* shuttle plasmid under control of the *pBlaF** promoter and fused to a short sequence encoding a six-histidine tag (Le Dantec *et al.*, 2001). Mt103 and MYC1558 were transformed with these plasmids, and the resulting transformants were grown to exponential phase in Sauton medium before fractionation. Proteins from the culture filtrate, cell wall and cytosol plus

membrane fractions were analysed by Western blot and the recombinant Plc proteins were revealed using an anti-His antibody. The analysis performed in the Mt103 and MYC1558 recombinant strains yielded similar results. The three recombinant enzymes were found to be associated with the cell wall fraction of *M. tuberculosis* Mt103 (Fig. 6). Interestingly, none of the Plc enzymes was detected in the culture filtrate, suggesting that phospholipases C remain associated with the cell envelope rather than being released into the culture medium.

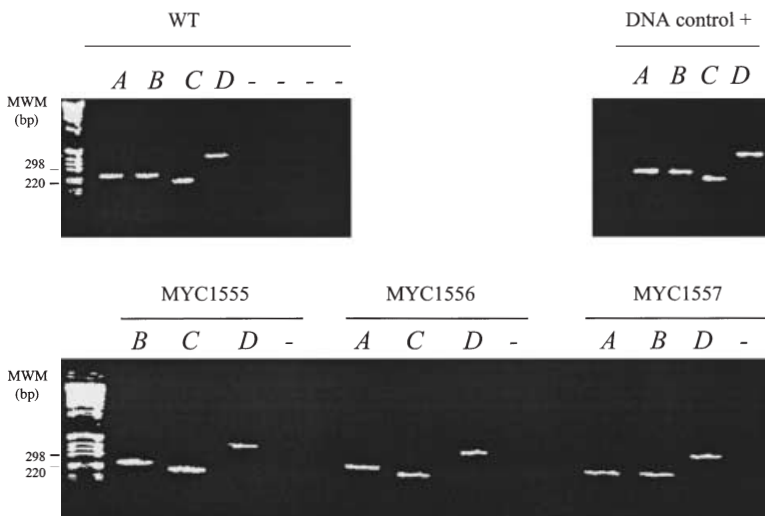


Fig. 4. Reverse transcription (RT)-PCR analysis of the *in vitro* expression of the *plc* genes in the MYC1555, MYC1556 and MYC1557 mutants. PCR products corresponding to the *plcA*, *plcB*, *plcC* and *plcD* genes were amplified using the sets of primers 23, 24, 25 and 26 respectively (Table 1). In the negative controls (-), the PCR reactions were performed directly on the RNA from the different strains to confirm the absence of DNA contamination. The negative controls (-) were performed with the primers specific to the *plcD* gene (26), which is present in the three mutant strains. The sets of primers 23, 24, 25 and 26 were used in the negative controls of Mt103.

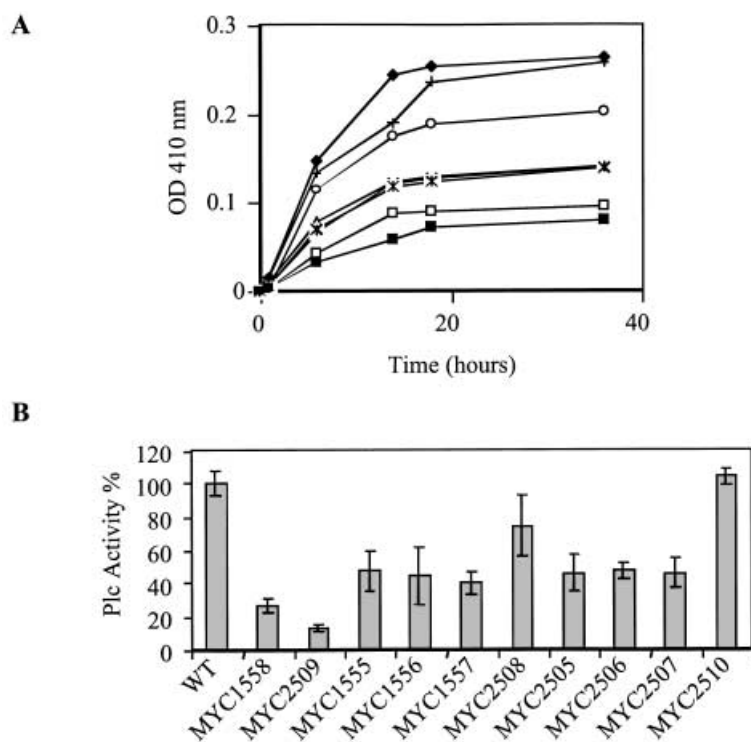


Fig. 5. Plc activities of the mutant and complemented mutant strains. Crude extracts of the different strains were incubated with *p*-nitrophenyl phosphorylcholine. The release of *p*-nitrophenol was monitored at 410 nm. A. Time-course of the Plc activity in *M. tuberculosis* Mt103 (◆), MYC1558 (□), MYC2509 (■), MYC1555 (△), MYC1556 (star), MYC1557 (▲), MYC2508 (○) and the complemented strain MYC2510 (×). B. Specific activities are expressed as the absorbance measured at 410 nm per mg of proteins. Activities are shown as a percentage of the activity detected in the wild-type cell extracts. The assays were carried out in triplicate.

In vitro and *in vivo* expression of the *plc* genes

The apparent redundancy among the four Plc enzymes, which share a high degree of sequence identity and have similar enzymatic activities, led us to question the role played by these four enzymes in the biology of *M. tuberculosis* during *in vitro* growth and host infection. We used a semiquantitative RT-PCR assay to determine the level of transcription of these genes under several conditions. The amount of cDNA produced, which is proportional to the amount of the specific transcript present in the original RNA sample, was measured.

In vitro, all of the *plc* genes were expressed (Fig. 4) which is consistent with the Plc activity assays shown in Fig. 5. The addition of phospholipids to the growth medium is known to stimulate phospholipase activity in bacteria. For example, specific acyl-hydrolysing phospholipase activities are six to 15-fold higher in cell extracts from *Mycobacterium microti* and *Mycobacterium avium* grown in the presence of phospholipids than in extracts from the same mycobacteria grown in the absence of phospholipids (Wheeler and Ratledge, 1992). Accordingly, the Plc activity detected in crude extracts of *M. tuberculosis* Mt103 and H37Rv increased four to 10-fold upon the addition of liposomes composed of phosphatidylcholine (PC) to the culture medium (Fig. 7A). We further investigated whether this increased Plc activity was related to an induction of the expression of the *plc* genes following the addition of PC. Total RNA was extracted from *M. tuberculosis* Mt103 and H37Rv that had

been grown in 7H9 medium with or without PC. We used a semiquantitative RT-PCR assay to compare the amount of mRNA corresponding to the *plc* genes recovered from *M. tuberculosis* H37Rv and Mt103 grown in the two culture conditions. The mean induction ratios ranged from 3 to 9 for the four *plc* genes (Fig. 7B), strongly suggesting that the induction of the expression of the *plc* genes in *M. tuberculosis* grown in the presence of PC is responsible for the higher enzymatic activity detected (Fig. 7A). There was no statistically significant difference between the induction ratio in *M. tuberculosis* H37Rv and that in Mt103 (Student's *t*-test, $p < 0.05$).

Phospholipids are major components of eukaryotic cell

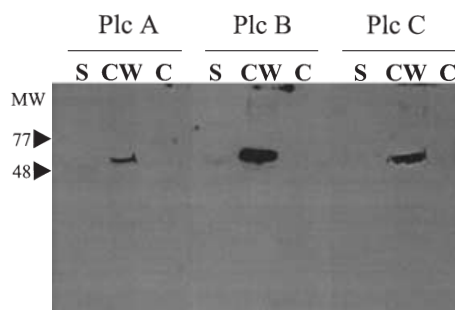


Fig. 6. Localization of recombinant His-tagged PlcA, PlcB and PlcC proteins in *M. tuberculosis*. Western blot analysis using anti-His antibodies. S, culture filtrate; CW, cell wall; C, cytosol plus membrane fractions of Mt103 grown in Sauton medium. 50 µg of proteins were loaded per well.

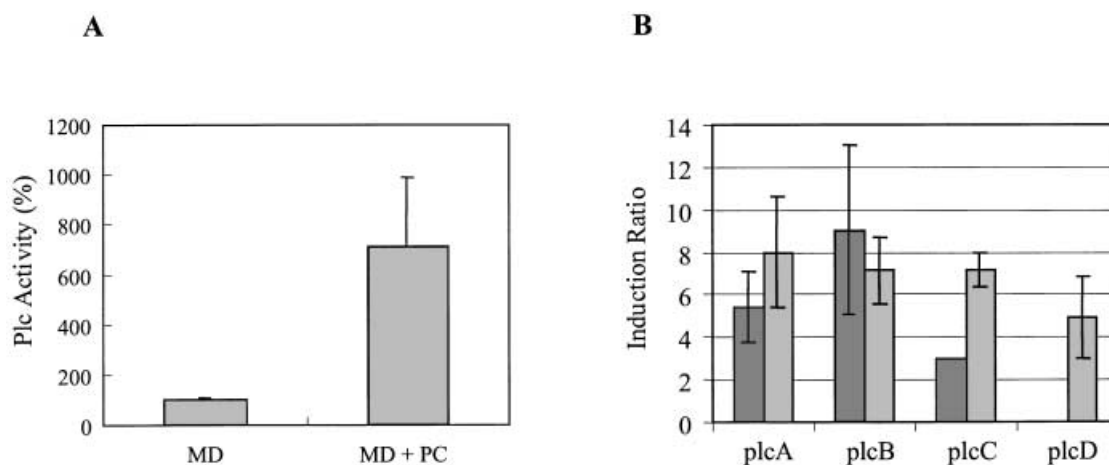


Fig. 7. Induction of the expression of the *plcA*, *plcB*, *plcC* and *plcD* genes in *M. tuberculosis* Mt103 and H37Rv after the addition of phosphatidylcholine (PC) to the culture medium.

A. Plc activity was measured in cell extracts of *M. tuberculosis* Mt103 grown in 7H9 medium (MD) or in 7H9 medium + 150 $\mu\text{g ml}^{-1}$ of phosphatidylcholine liposomes (MD + PC). The data represent the mean and standard deviations of values obtained from four different cultures.

B. Expression of the *plc* genes in *M. tuberculosis* H37Rv (black bars) and Mt103 (grey bars) grown in the presence or absence of PC liposomes in the culture medium. The induction ratio is the amount of transcripts, as measured by RT-PCR, detected in bacteria grown in the PC-supplemented 7H9 medium divided by the number of transcripts detected in bacteria grown in unsupplemented 7H9 medium. The data represent the mean and standard deviations of values obtained from two different cultures. RT-PCR was performed at least three times on each sample.

membranes. *M. tuberculosis* might be in close contact with high concentrations of these compounds when growing in the phagosomal compartment. Therefore, the *plc* genes are probably induced following the infection of host cells. To study the regulation of the *plc* genes within host cells, THP1 macrophages were infected with *M. tuberculosis* H37Rv (multiplicity of infection (MOI) = 10:1 bacteria per macrophage). The macrophages were lysed at different times after infection and the mRNAs corresponding to the *plc* genes were recovered. The recovered mRNAs were amplified using semiquantitative RT-PCR and their amounts were compared with those obtained from bacteria grown in axenic conditions (7H9 medium). In all experiments, *sigA* was used as an internal standard and the results are expressed relative to the amount of *sigA* transcripts. *sigA* is an essential housekeeping sigma factor in *M. tuberculosis*, and the amount of *sigA* mRNA remains constant in different growth conditions (Manganelli *et al.*, 1999) and during macrophage infection (Manganelli *et al.*, 2001). The expression of the *M. tuberculosis* H37Rv *plcA*, *plcB* and *plcC* genes was strongly induced immediately after infection (Fig. 8). This high level of induction was maintained for about 24 h and then rapidly decreased finally reaching the same level as detected *in vitro*. Interestingly, the maximum levels of induction occurred at different times for each gene, suggesting that the roles of the three genes are not redundant. *plcB* was significantly more induced than the other *plc* genes after 1 h of infection, whereas the *plcC* and *plcA*

genes were significantly more induced after 24 h of infection. Thus, the expression of the *plc* genes (relative to that of *sigA*) is greatly upregulated during the infection of host cells and their induction is transient. The differences

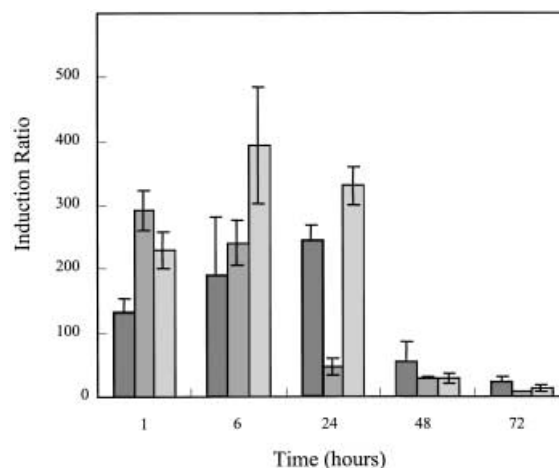


Fig. 8. Induction of the expression of the *M. tuberculosis* H37Rv *plcA*, *plcB* and *plcC* genes upon infection of THP-1 macrophages. The induction ratio is the amount of transcripts, as measured by RT-PCR, detected in bacteria grown in THP-1 macrophages divided by the number of transcripts detected in bacteria grown in 7H9 medium. In both conditions, the values are expressed relative to the amount of *sigA* transcripts. *plcA*, black bars; *plcB*, dark grey bars; *plcC*, light grey bars. The data represent the mean and standard deviations of values obtained from two different infected cell cultures. RT-PCR was performed at least three times on each sample.

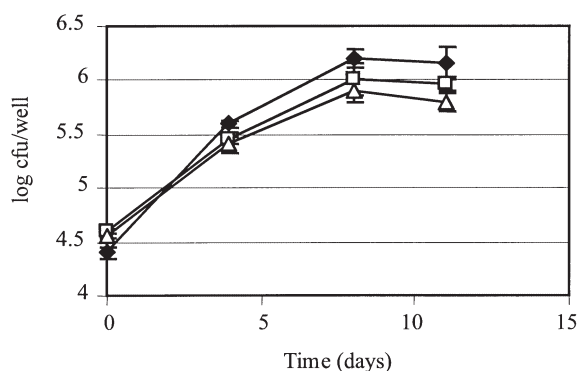
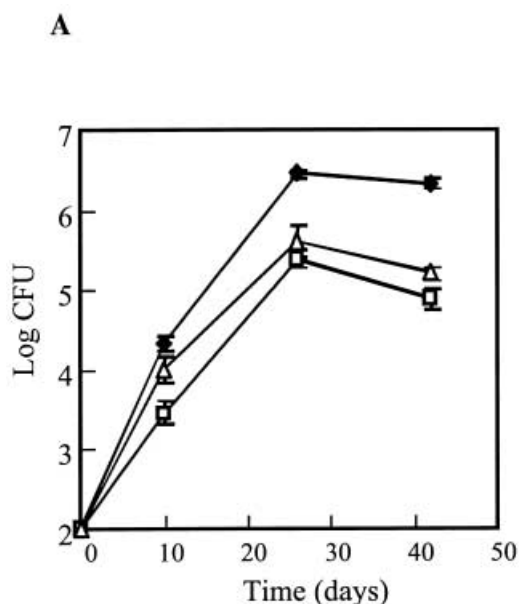


Fig. 9. Growth of *M. tuberculosis* Mt103, MYC1558 and MYC2509 in THP-1 derived macrophages. The MOI was 1:10 (bacterium per macrophages). The reported values represent the average and the standard deviation of data obtained from three independent wells. The experiment was repeated twice using independent stocks of bacteria and THP-1 cells. Mt103 (◆), MYC1558 (□) and MYC2509 (△).

between the induction ratios measured *in vitro* (using commercial PC) and *in vivo* may be due to differences in the quality or quantity of the phospholipid substrates or/and in the stability of the mRNAs.



The *plc* mutants do not show reduced growth in human macrophages

The finding that the expression of the *plc* genes was upregulated in *M. tuberculosis* after the infection of THP-1 macrophages suggests that these proteins contribute to the intracellular survival of the tubercle bacillus. To test this hypothesis, we infected the human monocytic cell line THP-1 with wild-type *M. tuberculosis* Mt103, the triple mutant (MYC1558) and the quadruple mutant (MYC2509) (Fig. 9). Macrophages were lysed at various times after infection and the number of intracellular viable *M. tuberculosis* colony-forming units (cfu) was determined. The mutants did not show any reduced virulence in this model.

Virulence of the mutant strains in the mouse model of infection

The virulence phenotypes of the triple (MYC1558) and quadruple (MYC2509) mutant strains were compared with that of the wild-type *M. tuberculosis* Mt103 strain in mice

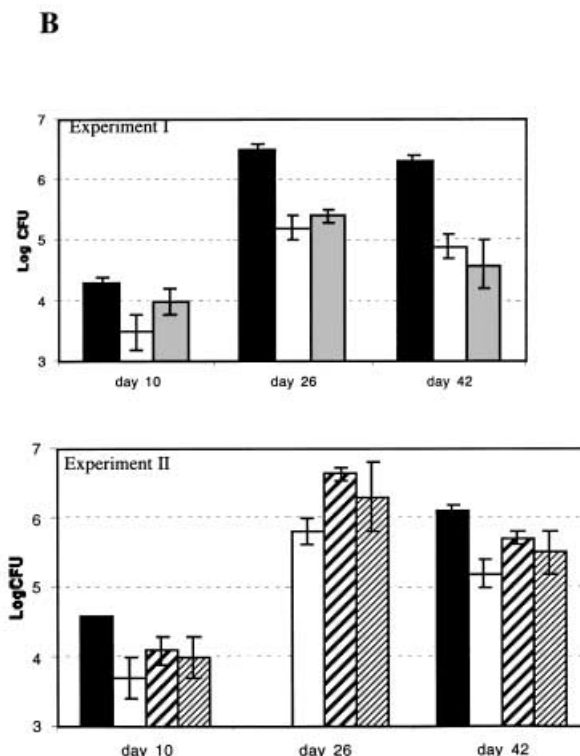


Fig. 10. Attenuation of the *M. tuberculosis* triple (MYC1558) and quadruple (MYC2509) *plc* mutants in mice infected via the aerosol route. A. Growth kinetics of wild-type Mt103 (◆), *M. tuberculosis* MYC1558 (□) and *M. tuberculosis* MYC2509 (△) in the lungs of mice. B. Growth kinetics of *M. tuberculosis* Mt103 (black bars), MYC1558 (white bars), and MYC1558 complemented with *plcA* (grey bars), complemented with *plcB* (thick hatched bars) and complemented with *plcC* (thin hatched bars) (MYC2505, MYC2506 and MYC2507 respectively) in the same model of infection and in the same organ. In experiment I, the growth kinetics of MYC2505 were compared with those of MYC1558 and Mt103. In experiment II, the growth kinetics of MYC2506 and MYC2507 were compared with those of MYC1558 and Mt103 (in experiment II, the Mt103 cfu could not be counted on day 26 because of plate contaminations). Error bars represent the standard deviations of the cfu counts obtained from four or five infected animals.

infected via the aerosol route. MYC1558 and MYC2509 were significantly attenuated for growth in the lungs of mice (Fig. 10A). At 26 days post infection, the mice infected with the mutants contained 10-fold less cfu than mice infected with the wild-type strain (Fig. 10A). The same reduction was observed in the spleen (data not shown). This difference increased to about 1.5 log units at later time-points (42 d), suggesting that phospholipases C have an important role in the persistence of infection. The quadruple mutant was not more attenuated than the triple mutant in this model. This suggests either that *plcD* does not contribute to virulence or that it acts in association with the other phospholipases. Infection experiments were also carried out with the MYC1558 strain complemented with the *plcA*, *plcB* or *plcC* genes carried on multicopy plasmids (Fig. 10B). Complementation with the *plcB* gene significantly increased the virulence of MYC1558 on days 26 and 42, whereas complementation with *plcA* increased the virulence of this strain on day 10. Complementation with *plcC* seemed to partially restore the virulence of the triple mutant on day 26, although the difference between the cfu counts of the two strains was not statistically significant, as determined by the Student's *t*-test (t_{95}). These results and the fact that the *plcABC* cluster is distant from other ORFs on the *M. tuberculosis* chromosome (there are 850 bp between the *plc* cluster and the upstream open reading frame (ORF), and 195 bp between the *plc* cluster and the downstream ORF) make it unlikely that the decreased virulence of the MYC1558 strain is due to polar effect of the mutation affecting the expression of neighbouring genes. Instead, these results demonstrate that *plcA*, *plcB* and *plcC* all contribute to the virulence of *M. tuberculosis*.

Discussion

The completion of the *M. tuberculosis* genome sequence revealed several genes thought to be involved in the virulence of the tubercle bacillus. These genes included the four putative phospholipase C genes. Two of these genes, *plcA* and *plcB*, were identified before the publication of the genome sequence of *M. tuberculosis* (Leão *et al.*, 1995). The other two, *plcC* and *plcD*, were identified after the completion of the *M. tuberculosis* genome sequence (Cole *et al.*, 1998). Despite the well known role of phospholipases C in the virulence of several intracellular bacterial pathogens, the involvement of these enzymes in the pathogenicity of mycobacteria had not yet been investigated.

The recent development of insertional mutagenesis tools for pathogenic mycobacteria (Pelicic *et al.*, 1997) allowed us to construct *M. tuberculosis* mutants inactivated in each of the four *plc* genes, a triple *plcABC* mutant and a quadruple *plcABCD* mutant. Plc activity assays

conducted on the non-complemented and complemented mutant strains revealed that the four *plc* genes encode functional phospholipases C, capable of hydrolysing a phosphatidylcholine-like substrate.

Reverse transcription (RT)-PCR assays provided evidence that the *plc* genes are induced during the infection of human THP-1-derived macrophages. This was not unexpected as Wheeler and Ratledge (Wheeler and Ratledge, 1992) showed that phospholipase activities are higher in mycobacteria grown in mice than in those grown in the lipid-free Dubos medium. The expression of the *plc* genes of other pathogenic bacteria is also upregulated during host infection (Agaisse *et al.*, 1999; Marquis and Hager, 2000). The fact that the expression of the *M. tuberculosis* *plc* genes is upregulated in macrophages suggests that phospholipases C play a role in host infection. Consistent with this hypothesis, the disruption of the *plcABCD* or *plcABC* genes impaired the ability of *M. tuberculosis* Mt103 to multiply in the lungs and spleen of infected mice. This is the first evidence that phospholipases C are required for the full virulence of *M. tuberculosis*. The attenuated phenotype of the mutants only became evident after the acute phase of the infection, suggesting that phospholipases C are important during persistent infection. Virulence was partially restored when the triple *plcABC* mutant was complemented with each of the *plc* genes, *plcA*, *plcB* or *plcC*. Interestingly, the virulence did not decrease further in the quadruple mutant, suggesting that PlcD does not make a significant contribution to the virulence of *M. tuberculosis* or that it acts in synergy with the other Plc enzymes. A limited role for PlcD in the virulence of the tubercle bacillus would be consistent with the finding of Gordon and colleagues (Gordon *et al.*, 1999) who showed that three out of seven clinical isolates analysed were deficient in the expression of *plcD*. Interestingly, the triple and quadruple mutants were not attenuated in infected THP-1 cells despite the fact that the expression of the *plc* genes was highly induced in this model. The growth kinetics in this cellular model may not have been measured for a sufficient period of time to detect the effects of the mutations on the intracellular multiplication and survival of *M. tuberculosis*. Furthermore, *in vitro* macrophage cultures are incomplete models that do not reflect the exact conditions encountered by bacteria *in vivo* and therefore may not have allowed us to detect certain attenuated phenotypes.

As mentioned earlier, the *plcA*, *plcB* and *plcC* genes are absent from the genomes of *M. bovis* and *M. bovis* BCG, which only carry a full-length copy of the *plcD* gene (Gordon *et al.*, 1999). As PlcD exhibits some phospholipase C activity in *M. tuberculosis*, this enzyme may account for the Plc activity detected in *M. bovis* (Johansen *et al.*, 1996). The absence of Plc activity in *M. bovis* BCG could be due to defects in the expression of the *plcD*

gene. Alternatively, the PlcD enzyme may not be functional in *M. bovis* and *M. bovis* BCG, and other unrelated phospholipases C or other enzymes (such as a phospholipase D and phosphatases) may account for the Plc activity detected in *M. bovis*.

The fact that *M. tuberculosis* contains three or four phospholipases C that are important for its virulence in mice raises the questions about the functional redundancy of these enzymes. As noted earlier, other organisms, such as *L. monocytogenes* (Mengaud *et al.*, 1991; Raveneau *et al.*, 1992), contain two Plc enzymes that hydrolyse different substrates. Therefore, the *M. tuberculosis* Plc enzymes may have different affinities for different phospholipid substrates, thereby increasing their spectrum of action. As suggested by Johansen and colleagues (Johansen *et al.*, 1996), a complementary hypothesis is that the *plc* genes are regulated differently so that they act at different stages of host infection. The differential temporal patterns of activation of the *plcA*, *plcB* and *plcC* genes during cell infection may support this last hypothesis (Fig. 8).

Phospholipases C expressed within host cells might serve several functions related to virulence. First, they might provide the bacteria with nutrients. Indeed, biochemical studies suggest that in chronically infected lung tissues, fatty acids might be a major source of carbon and energy for *M. tuberculosis* metabolism (Segal, 1984; Wheeler *et al.*, 1990). The relatively high phospholipase activities detected in mycobacteria harvested from host tissues (Wheeler and Ratledge, 1991; 1992) and the induction of the *M. tuberculosis plc* genes upon entry into phagocytic cells (Fig. 8) are consistent with these enzymes having a role in the release of fatty acids from host phospholipids. *M. tuberculosis* may then use the fatty acids as a carbon source through the β -oxidation cycle and the glyoxylate shunt. In this regard, it is interesting to compare the regulation of the *plc* genes with that of the isocitrate lyase gene (*icl*) which encodes an essential glyoxylate shunt enzyme that is expected to act downstream of the phospholipases C. In *M. tuberculosis*, the expression of *icl* is strongly induced during the first few hours of macrophage infection, before returning to background levels 24 h post infection (McKinney *et al.*, 2000). This is similar to the pattern observed for phospholipases C genes, which suggests that they both have roles in the same pathway. Furthermore, as for the *plc* genes, disruption of the *icl* gene impaired the ability of *M. tuberculosis* to multiply and to persist in mouse organs during persistent infection.

A second possible role for phospholipases C may be to degrade the phagosomal membrane, thus modifying its permeability or leading to total degradation. However, unlike the situation in other pathogenic bacteria (Marquis *et al.*, 1995), mycobacterial phospholipases C remain

associated with the cell envelope (Fig. 6) (Wheeler and Ratledge, 1991) and are not released into the culture medium. This localization appears to contradict the role of these enzymes in the degradation of the phagosomal membrane. Some authors have speculated that this arrangement indicates that mycobacterial phospholipases have a non-aggressive role (Wheeler and Ratledge, 1992) which may ultimately allow the controlled release of fatty acids from the host allowing intracellular mycobacteria to obtain nutrients without causing major damage. This property would be advantageous to mycobacterial agents that cause chronic diseases.

Finally, by activating the arachidonic acid cascade, *M. tuberculosis* phospholipases C may interfere with signal transduction events in infected cells, thus modulating the host immune responses (Meyers and Berk, 1990; Titball, 1993).

In conclusion, this report provides the first evidence that phospholipases C are involved in the virulence of *M. tuberculosis*. Based on the similarities between the *plc* genes and the *icl* gene in terms of intracellular regulation and their involvement in the late phase of mouse infection, we propose that the major role of phospholipases C in the course of infection is to provide *M. tuberculosis* with host fatty acids, which are then used as a carbon source through the β -oxidation cycle and the glyoxylate shunt.

Experimental procedures

Bacterial strains and growth conditions

Mycobacterium smegmatis mc²155 (Snapper *et al.*, 1990), *Mycobacterium bovis* BCG Pasteur (CIPT 140040001) and *Mycobacterium tuberculosis* 103 (clinical isolate Mt103) were used in this study. Mycobacteria were grown at 32°C, 37°C or 39°C in liquid Middlebrook 7H9 medium (Difco) supplemented with 0.05% Tween 80 and ADC (Becton Dickinson), in Sauton medium or on solid Middlebrook 7H10 or 7H11 medium (Difco) supplemented with OADC (Becton Dickinson). *Escherichia coli* DH5 α , the strain used in the cloning experiments, was grown on Luria–Bertani medium (LB) (Difco). When required, the medium was supplemented with 2% sucrose or the following amounts of antibiotics: 100 μ g ml⁻¹ of ampicillin, 100 μ g ml⁻¹ of gentamicin, 20 μ g ml⁻¹ of kanamycin, 200 μ g ml⁻¹ of hygromycin for *E. coli* or 50 μ g ml⁻¹ for mycobacteria.

Construction of the *plcABC*, *plcD* and *plcABCD* mutants

plcD (MYC2508), *plcABC* (triple mutant, MYC1558) and *plcABCD* (quadruple mutant, MYC2509) mutants were constructed by allelic replacement using the Ts/*sacB* method described by Pelicic and colleagues (Pelicic *et al.*, 1997). *plcABC::km*, the disrupted *plcABC* allele used in the gene replacement experiment, was generated by polymerase chain reaction (PCR) using the primer pairs 7/8 and 9/10

(Table 1). The amplified PCR fragments (a 1.5 kb fragment containing the first 915 bp of *plcA* and the upstream region, and a 1.8 kb fragment carrying the last 1330 bp of *plcC* and the downstream region) were digested with *XhoI* and ligated to generate a disrupted *plcABC* cluster lacking a 2.6 kb internal DNA fragment. The disrupted *plcABC* fragment was then cut with *EcoRI* and inserted into *EcoRI*-cut pUC19 yielding pUCP. The kanamycin resistance cassette (*km*) from pUC4K (Amersham Pharmacia Biotech) was cut with *SalI* and then introduced into the unique *XhoI* site of pUCP. *plcABC::km* carried on a 4.5 kb *EcoRI* fragment was then inserted into *EcoRI*-cut pXYL4 (Pelicic *et al.*, 1997), yielding pPKX. Finally, p27PKX, the vector used for allelic replacement, was obtained by inserting the 5.5 kb *BamHI* fragment from pPKX carrying *plcABC::km* and *xyIE* into the *BamHI* site of pPR27 (Pelicic *et al.*, 1997). *plcD::hyg*, the disrupted allele used for allelic replacement at the *plcD* locus of *M. tuberculosis* was generated by PCR. A 750 bp DNA fragment designed to carry a *NotI* and a *HindIII* restriction sites and a 1000 bp fragment designed to carry a *HindIII* and a *SpeI* restriction sites were amplified by PCR using the 11/12 and 13/14 primer pairs, respectively (see Table 1). The two *HindIII*-cut PCR fragments were ligated and the resulting 1.75 kb fragment was digested with *NotI* and *NdeI* before insertion into a *NotI*/*SpeI*-cut derivative of pBluescript KS⁻ (Stratagene) devoid of *HindIII* site yielding pBS(*plcD*). A hygromycin resistance cassette (*hyg*) extracted from pUCHygro on a *HindIII* restriction fragment was then ligated into the *HindIII*-cut pBS(*plcD*) to yield pBS(*plcD::hyg*). pCR8, the plasmid used for allelic replacement at the *plcD* locus of Mt103 and MYC1558 was finally obtained by inserting the *NotI*-*SpeI*-cut disrupted *plcD::hyg* allele from pBS(*plcD::hyg*) into pPR23 (Pelicic *et al.*, 1997), which had been cut with the same enzymes.

DNA manipulations and PCR amplifications

All nucleic acid manipulations were performed according to standard molecular biology techniques (Sambrook *et al.*, 1989) or to the recommendations of the manufacturers. All transformations were performed by electroporation using a Gene Pulser unit (Bio-Rad). Plasmids were extracted from *E. coli* using the QIAprep Spin Miniprep kit and DNA fragments were purified using the QIAquick PCR-purification kit and the QIAquick gel Extraction kit (Qiagen).

Polymerase Chain Reactions amplifications were carried out in a GenAmp PCR system 9600 machine (Perkin-Elmer). The primers used are described in Table 1. The PCR mixes (GenAmpR PCR core reagents, Roche) were as recommended by the manufacturer with the following modifications: dNTP were included at a final concentration of 100 μ M, 1 unit of AmpliTaq Gold DNA polymerase or Turbo Pfu DNA polymerase (Stratagene) and 20 pmol of each primer were used per reaction. The PCR conditions consisted of one denaturation cycle (95°C, 10 min for AmpliTaq Gold or 95°C, 5 min for Turbo Pfu DNA polymerase), followed by 30 cycles of denaturation (95°C, 1 min), annealing (60°C, 1 min), primer extension (72°C, 1.5 min) and a final extension at 72°C for 10 min.

The labelling of the DNA probes with [α -³²P]-dCTP and Southern blot analyses were performed as described (Jackson *et al.*, 1997).

Construction of the complementation vectors carrying the *plcA*, *plcB* and *plcC* genes

Wild-type copies of the Mt103 *plcA*, *plcB* and *plcC* genes were PCR-amplified using the 15/16, 17/18 and 19/20 primer pairs respectively (Table 1). The primers were designed to generate PCR products harbouring *BamHI* and *KpnI* restriction sites at their extremities. The amplification products were ligated into the *BamHI* and *KpnI*-cut pMIP12 (Le Dantec *et al.*, 2001), a *Mycobacterium/E. coli* replicative shuttle plasmid that allows genes to be expressed under the control of the *pBlaF** promoter and C-terminal six-His-tagged recombinant proteins to be produced. *E. coli* DH5 α transformants harbouring pMIP12 vectors with *plcA*, *plcB* or *plcC* inserts (named pCR1, pCR2 and pCR3 respectively) (Table 2) were screened by colony hybridization as described by Jackson and colleagues (Jackson *et al.*, 1997) with minor modifications. The membranes were washed in high-stringency conditions at 65°C, twice in 2 \times SSC plus 0.1% SDS, once in 1 \times SSC plus 0.1% SDS and twice in 0.1 \times SSC plus 0.1% SDS. The probes used corresponded to the PCR-amplified *plcA*, *plcB* and *plcC* genes described above. For the complementation of MYC1558 with the *plcA*, *plcB* and *plcC* genes, the three structural genes were extracted from pCR1, pCR2 and pCR3 on *XbaI*-*BglII* restriction fragments and inserted into *XbaI*- and *BglII*-cut pMIP12H, a derivative of pMIP12 carrying a hygromycin resistance cassette. pCR9, the vector carrying the entire *plcABC* cluster was constructed by inserting a blunt-ended 5497 bp *MseI*-*BlnI* restriction fragment from cosmid MTCY98 into the *XbaI*-cut and blunt-ended pIPX59, a *Mycobacterium* integrative vector harbouring a hygromycin resistance cassette (Berthet *et al.*, 1998).

DNA sequencing

Double-stranded plasmid DNA (pCR1, pCR2 and pCR3) and PCR fragments were sequenced using an automated DNA sequencer (Applied Biosystems, model 373) with a dye deoxy terminator cycle sequencing kit (Applied Biosystems).

Extraction of RNA from *M. tuberculosis* and RT-PCR experiments

Mycobacterium tuberculosis was grown to OD₆₀₀ = 0.6. Cells were broken in a solution of Trizol (1 ml) (Life Technologies) with mini glass beads using a Bead Beater apparatus (Poly-labo) set at maximum speed. RNA was extracted with 300 μ l of chloroform:isoamyl alcohol. After 10 min of centrifugation at 13000 *g*, the aqueous phase was transferred to a tube containing 270 μ l of isopropanol. Total RNA was then precipitated overnight at 4°C and washed with 1 ml of a 75% ethanol solution before resuspension in diethyl pyrocarbonate (DEPC, Sigma)-treated water. Contaminating DNA was removed by digestion with DNase I according to the manufacturer's instructions (Ambion). The same protocol was used to extract RNA from *M. tuberculosis* infecting THP-1 cells. At different time-points, infected macrophages were treated with Trizol before RNA extraction. This experiment was repeated twice on two independent stocks of THP-1 cells.

Reverse transcription (RT)-PCR experiments were re-

peated at least three times on the RNA extracted from infected THP-1 cells and carried out as described by Manganelli and collaborators (Manganelli *et al.*, 1999) using 2 µg of RNA and specific primers corresponding to each *plc* gene and to the *sigA* gene (Table 1). The PCR conditions were the same as those used by Manganelli and colleagues (Manganelli *et al.*, 1999). The *plc* genes gave single specific amplification products that could be labelled with the general fluorescent probe Sybr Green (Sigma), 25 ng µl⁻¹ final concentration. The amplification of *sigA* by the Sybr Green technique was less specific and clearly sensitive to RNA contamination from the macrophages. Therefore, a specific fluorescent probe (beacon, 25 ng µl⁻¹ final concentration) was used for *sigA* (Table 1). The significance of differences was determined by the Student's *t*-test (t_{95}).

Fractionation of *M. tuberculosis*

Culture filtrates from *M. tuberculosis* cultures grown in Sauton medium were filtered twice through a 0.2 µm membrane (Millipore) to remove contaminating cells. Bacterial cells were resuspended in water and broken for 3 min with mini glass beads in a Bead Beater apparatus (Polylabo) set at maximum speed. Beads and unbroken cells were removed by centrifugation at 5000 *g* for 10 min. Supernatants were further centrifuged for 30 min at 15 000 *g*. The resulting supernatant contained cytoplasmic and membrane components and the pellet contained cell wall components. The protein concentration of each fraction was measured using a Coomassie blue assay (Bio-Rad). In total, 100 µg of proteins was used for the isocitrate dehydrogenase activity assay as described previously (Raynaud *et al.*, 1998). Isocitrate dehydrogenase is a cytosolic enzyme (Andersen *et al.*, 1991) that can be used as a marker to check that the cell wall and culture filtrate fractions are not contaminated with cytoplasm.

Phospholipase C activity

The substrate used in the phospholipases C assays was the chromogenic derivative of phosphatidylcholine *p*-nitrophenyl phosphorylcholine (*p*NPPC, Sigma) (Kurioka and Matsuda, 1976). Assays were performed on crude bacterial extracts consisting of the supernatant recovered after breaking *M. tuberculosis* cells in a Bead Beater and centrifugation at 5000 *g* for 10 min. Crude extracts containing 500 µg of proteins were incubated at 37°C for 1, 6, 14, 18 and 36 h in a buffer containing 6 mM Tris (pH 7.2), 5 mM *p*NPPC and 1.5% sorbitol. The reaction was stopped by the addition of 3 ml of a 1% Na₂CO₃ solution and the release of *p*-nitrophenol was measured at 410 nm. Negative controls contained water instead of cell extracts and positive controls contained commercial phospholipase C (Sigma). To avoid bacterial contamination in the reaction mix, chloramphenicol (100 µg ml⁻¹) and gentamicin (100 µg ml⁻¹) (Sigma) were systematically added. The presence of possible microbial contamination was carefully checked after each long incubation period (Raynaud *et al.*, 1998). All enzymatic assays were performed in triplicates. The significance of differences was determined by the Student's *t*-test (t_{95}).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on a Mini-Protean apparatus (Bio-Rad) and proteins were transferred to Hybond C membranes (Amersham) with a mini-Transblot apparatus (Bio-Rad) following the recommendations of the manufacturer. The production of recombinant Plc proteins by *M. tuberculosis* recombinant strains was checked using a mouse monoclonal anti-His antibody (Qiagen) diluted 1:3000 and a sheep anti-mouse IgG HRP conjugated secondary antibody (Amersham Pharmacia Biotech) diluted 1:10 000. Bound antibodies were detected using the ECL system (Amersham Pharmacia Biotech).

Aerosol infection in mice

Six- to eight-week-old female BALB/c mice were infected with approximately 200 cfu of *M. tuberculosis* Mt103, MYC1558, MYC2509, MYC2501, MYC2502 and MYC2503 via the aerosol route. *M. tuberculosis* aerosols were generated from bacterial suspensions consisting of 3 × 10⁷ cfu ml⁻¹ in phosphate-buffered saline (PBS) solution (pH 7.4) with 0.05% Tween 80. Mice were exposed to the aerosols for 15 min. Four or five mice were used for each experimental time-point. At various time-points post infection, the lungs and spleens were removed aseptically and homogenized. Serial dilutions of organ homogenates were plated on solid medium 7H11 supplemented with the appropriate antibiotics (Jackson *et al.*, 1999).

Infection of THP-1-derived macrophages with *M. tuberculosis*

THP-1 cells were obtained from the ATCC collection and grown at 37°C in a 5% CO₂ atmosphere. Cells were maintained in RPMI-1640 medium (Life Technologies) containing 2 mM L-glutamine, 1.5 g l⁻¹ of sodium bicarbonate, 4.5 g l⁻¹ of glucose, 1.0 mM sodium pyruvate, 50 µM 2-mercaptoethanol and 10% foetal bovine serum. THP-1 cell suspensions were adjusted to a concentration of 10⁶ cells ml⁻¹ in warm RPMI supplemented with 50 nM phorbol 12-myristate 13-acetate (Sigma), used to seed tissue culture plates (1 ml per well) and allowed to differentiate for 24 h. The medium was then removed and replaced with 1 ml of bacterial suspension in RPMI containing 1 × 10⁵ cfu ml⁻¹ (multiplicity of infection (MOI) = 1:10 cfu per macrophage). After 16 h at 37°C, the medium was removed and the wells were washed twice with RPMI to remove extracellular bacteria. On days 1, 4, 7 and 11, cells were lysed with 500 µl of a 2% saponin solution, and the number of viable intracellular cfu was counted by plating serial dilutions of the lysis solution onto Middlebrook 7H10 agar. This infection experiment was carried out in duplicate.

Acknowledgements

We thank Pierre Chavarro and Eddy Maranghi for their technical help with the infection of mice. We would also like to thank Dr Eugénie Dubnau, Dr Patricia Fontan and Mr Salvatore Marras for their excellent advice and for their help

with the RT-PCR experiments. We are grateful to Dr Nathalie Winter for the gift of pMIP12 and pMIP12H, to Pr. Stewart Cole for cosmid MTCY98, to Dr Jean-Marc Reytrat for excellent discussions and to Dr Philip Draper for critical reading of the manuscript. This work was supported by grants from the European Commission (contract IC18-CT97-0252) and from the European Economic Community (contract QLK2-2000-01761). I.S. was supported by the NIH grant AI 44856.

References

- Agaisse, H., Gominet, M., Økstad, O.A., Kolstø, A.B., and Lereclus, D. (1999) PlcR is a pleiotropic regulator of extracellular virulence factor gene expression in *Bacillus thuringiensis*. *Mol Microbiol* **32**: 1043–1053.
- Andersen, P., Askgaard, D., Ljungqvist, L., Bennedsen, J., and Heron, I. (1991) Proteins released from *Mycobacterium tuberculosis* during growth. *Infect Immun* **59**: 1905–1910.
- Berthet, F.-X., Lagranderie, M., Gounon, P., Laurent-Winter, C., Ensergueix, D., Chavarot, P., et al. (1998) Attenuation of virulence by disruption of the *Mycobacterium tuberculosis* *erp* gene. *Science* **282**: 759–762.
- Camilli, A., Goldine, H., and Portnoy, D.A. (1991) *Listeria monocytogenes* mutants lacking phosphatidylinositol-specific phospholipase C are avirulent. *J Exp Med* **173**: 751–754.
- Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., et al. (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**: 537–544.
- Gomez, A., Mve-Obiang, A., Vray, B., Remacle, J., Chemlal, K., Meyers, W.M., et al. (2000) Biochemical and genetic evidence for phospholipase C activity in *Mycobacterium ulcerans*. *Infect Immun* **68**: 2995–2997.
- Gordon, S.V., Brosch, R., Billault, A., Garnier, T., Eiglmeier, K., and Cole, S.T. (1999) Identification of variable regions in the genomes of tubercle bacilli using bacterial artificial chromosome arrays. *Mol Microbiol* **32**: 643–655.
- Jackson, M., Portnoi, D., Catheline, D., Dumail, L., Rauzier, J., Legrand, P., and Gicquel, B. (1997) *Mycobacterium tuberculosis* Des protein: an immunodominant target for the humoral response of tuberculosis patients. *Infect Immun* **65**: 2883–2889.
- Jackson, M., Raynaud, C., Lanéelle, M.A., Guilhot, C., Laurent-Winter, C., Ensergueix, D., et al. (1999) Inactivation of the antigen 85C gene profoundly affects the mycolate content and alters the permeability of the *Mycobacterium tuberculosis* cell envelope. *Mol Microbiol* **31**: 1573–1587.
- Johansen, K.A., Gill, R.E., and Vasil, M.L. (1996) Biochemical and molecular analysis of phospholipase C and phospholipase D activity in *Mycobacteria*. *Infect Immun* **64**: 3259–3266.
- Kurioka, S., and Matsuda, M. (1976) Phospholipase C assay using *p*-nitrophenylphosphorylcholine together with sorbitol and its application to studying the metal and detergent requirement of the enzyme. *Anal Biochem* **75**: 281–289.
- Le Dantec, C., Winter, N., Gicquel, B., Vincent, V., and Picardeau, M. (2001) Genomic sequence and transcriptional analysis of a 23-kilobase mycobacterial linear plasmid; evidence for horizontal transfer and identification of plasmid maintenance systems. *J Bacteriol* **183**: 2157–2164.
- Leão, S.C., Rocha, C.L., Murilla, L.A., Parra, C.A., and Patarroyo, M.E. (1995) A species-specific nucleotide sequence of *Mycobacterium tuberculosis* encodes a protein that exhibits hemolytic activity when expressed in *Escherichia coli*. *J Bacteriol* **178**: 1274–1282.
- Manganelli, R., Dubnau, E., Tyagi, S., Kramer, F.R., and Smith, I. (1999) Differential expression of 10 sigma factor genes in *Mycobacterium tuberculosis*. *Mol Microbiol* **31**: 715–724.
- Manganelli, R., Voskuil, M.I., Schoolnik, G.K., and Smith, I. (2001) The *Mycobacterium tuberculosis* ECF sigma factor σ^E role in global gene expression and survival in macrophages. *Mol Microbiol* **41**: 423–437.
- Marquis, H., and Hager, E.J. (2000) pH-regulated activation and release of a bacteria-associated phospholipase C during intracellular infection by *Listeria monocytogenes*. *Mol Microbiol* **35**: 289–298.
- Marquis, H., Doshi, V., and Portnoy, D.A. (1995) The broad-range phospholipase C and a metalloprotease mediate listeriolysin-O-independent escape of *Listeria monocytogenes* from a primary vacuole in human epithelial cells. *Infect Immun* **63**: 4531–4534.
- McKinney, J.D., Höner zu Bentrup, K., Muñoz-Elias, E.J., Miczak, A., Chen, B., Chan, W.T., et al. (2000) Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature* **406**: 735–738.
- McNamara, P.J., Bradley, G.A., and Songer, J.G. (1994) Targeted mutagenesis of the phospholipase D gene results in decreased virulence of *Corynebacterium pseudotuberculosis*. *Mol Microbiol* **12**: 921–930.
- Mengaud, J., Braun-Breton, C., and Cossart, P. (1991) Identification of phosphatidylinositol-specific phospholipase C active in *Listeria monocytogenes*: a novel type of virulence factor. *Mol Microbiol* **5**: 367–372.
- Meyers, D.J., and Berk, R.S. (1990) Characterization of phospholipase C from *Pseudomonas aeruginosa* as a potent inflammatory agent. *Infect Immun* **58**: 659–666.
- Pellicic, V., Jackson, M., Reytrat, J.M., Jacobs, W.R., Jr, Gicquel, B., and Guilhot, C. (1997) Efficient allelic exchange and transposon mutagenesis in *Mycobacterium tuberculosis*. *Proc Natl Acad Sci USA* **94**: 10955–10960.
- Raveneau, J., Geoffroy, C., Beretti, J.L., Gaillard, J.L., Alouf, J.E., and Berche, P. (1992) Reduced virulence of a *Listeria monocytogenes* phospholipase-deficient mutant obtained by transposon insertion into the zinc metalloprotease gene. *Infect Immun* **60**: 916–921.
- Raynaud, C., Etienne, G., Peyron, P., Lanéelle, M.A., and Daffé, M. (1998) Extracellular enzyme activities potentially involved in the pathogenicity of *Mycobacterium tuberculosis*. *Microbiol* **144**: 577–587.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
- Segal, W. (1984) Growth dynamics of *in vivo* and *in vitro* grown mycobacterial pathogens. In *The Mycobacteria: a Sourcebook*. Kubica, G.P., and Wayne, L.G. (eds). New York: Dekker, pp. 547–573.

- Snapper, S.B., Bloom, B.R., and Jacobs, J. R. (1990) Molecular genetic approaches to mycobacterial investigation. In: *Molecular Biology of the Mycobacteria*. McFadden, J. (ed.). London: Surrey University Press, pp. 199–218.
- Songer, J.G. (1997) Bacterial phospholipases and their role in virulence. *Trends Microbiol* **5**: 156–161.
- Steenken, W., and Gardner, L.U. (1946) History of H37 strain of tubercle bacillus. *Am Rev Tuberc* **54**: 62–66.
- Terada, L.S., Johansen, K.A., Nowbar, S., Vasil, A.I., and Vasil, M.L. (1999) *Pseudomonas aeruginosa* hemolytic phospholipase C suppresses neutrophil respiratory burst activity. *Infect Immun* **67**: 2371–2376.
- Titball, R.W. (1993) Bacterial phospholipases C. *Microbiol Rev* **57**: 347–366.
- Wheeler, P.R., and Ratledge, C. (1991) Phospholipase activity of *Mycobacterium leprae* harvested from experimentally infected Armadillo tissue. *Infect Immun* **59**: 2781–2789.
- Wheeler, P.R., and Ratledge, C. (1992) Control and location of acyl-hydrolysing phospholipase activity in pathogenic mycobacteria. *J Gen Microbiol* **138**: 825–830.
- Wheeler, P.R., Bulmer, K., and Ratledge, C. (1990) Enzymes for biosynthesis *de novo* and elongation of fatty acids in mycobacteria: is *Mycobacterium leprae* competent in fatty acid biosynthesis? *J Gen Microbiol* **136**: 211–217.