UNIVERSIDADE DE LISBOA
FACULDADE DE FARMÁCIA

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DOUTORAMENTO EM FARMÁCIA
MICROBIOLOGIA

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Análise funcional do gene *lys*B do módulo de lise do micobacteriófago Ms6

Dissertação apresentada à Faculdade de Farmácia da Universidade de Lisboa para obtenção do grau de Doutor em Farmácia (Microbiologia)

Ana Filipa Morais Esteves Gil
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À Carolina

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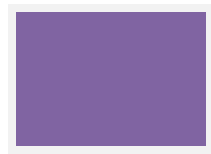


SUMMARY

Most described bacteriophages end their replication cycle by lysing their hosts. This characteristic has attracted researchers all over the world aiming to develop new alternatives to fight bacterial infectious diseases. All double stranded DNA phages achieve lysis by synthesizing two essential proteins: an endolysin, a protein with peptidoglycan hydrolase activity, and a holin, a small membrane protein that disrupts the cytoplasmic membrane and allows the access of the endolysin to its target or its activation. Holins have been described as essential to determine the optimum timing of lysis, so that the phage release is productive for phage survival. In addition to the endolysin and holin genes, new genes have been identified in the lysis cassette of bacteriophages that infect Gram-negative bacteria. These genes, exemplified by the λ Rz and Rz1 encoded proteins that compromise the stability of the outer membrane, eliminating the last barrier to the release of the progeny virions. No homologues of *Rz/Rz1* genes have been identified in mycobacteriophage genomes. The lytic cassette of mycobacteriophage Ms6, a phage that infects *Mycobacterium smegmatis* cells, comprises, in addition to an endolysin and a holin, three accessory lysis proteins encoded by genes *gp1*, *gp3* (*lys*B) and *gp5*. Despite being classified as Gram-positive bacteria, mycobacteria have a complex cell wall. This cell wall consists of peptidoglycan covalently linked to arabinogalactan, which is in turn esterified to a variety of long chain (C_{60}–C_{90}), α-branched, β-hydroxy fatty acids (mycolic acids).

The present work describes the characterization of the protein encoded by the mycobacteriophage Ms6 gene *lys*B and its role in mycobacteria lysis. A BLAST search within the sequences of protein databases revealed similarities to other putative proteins encoded by mycobacteriophages. After His6-LysB protein production in *E. coli*, it was possible to test its activity in several lipolytic enzymes substrates. Bioinformatics analysis revealed the presence of a conserved motif (GYSQG), characteristic of enzymes with lipolytic activity. The results show that LysB is indeed a lipolytic enzyme showing higher affinity towards substrates of longer chain length (C_{16} and C_{18}). It is demonstrated that the natural substrates of LysB are mycobacterial lipids containing mycolic acids and that that its main target is mycolyl-arabinogalactan peptidoglycan complex, as it was shown to release mycolic acids from mAGP. Additionally, LysB is also able to hydrolyze the trehalose 6,6'-dimycolate (TDM) from both fast and slow growing mycobacteria species. Construction of a mycobacteriophage Ms6 Δ*lys*B demonstrated that the gene product of *lys*B is nonessential for phage viability, but is involved in the lysis mechanism. Given the complexity of the mycobacterial cell envelope, it is easy to understand why mycobacteriophages have evolved new lysis strategies by acquiring them through their evolution.

Key words: Mycobacteriophage Ms6, phage lysins, lipolytic enzymes; mycolic acids

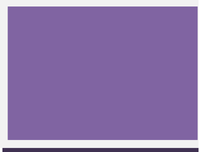
SUMÁRIO

A maioria dos bacteriófagos descritos na literatura são capazes de terminar um ciclo de replicação lisando as suas células hospedeiras. Esta característica tem atraído os investigadores no sentido de explorar esta propriedade para desenvolver novas alternativas para combater doenças infecciosas de origem bacteriana. Todos os bacteríofagos de cadeia dupla de DNA atingem a lise através da síntese de 2 proteínas essenciais: uma endolisina, uma proteína com actividade de hidrólise, e uma holina. Proteína de pequenas dimensões que destrói a membrana citoplasmática permitindo que a endolisina aceda ao seu substrato ou seja activada. As holinas estão descritas como sendo essenciais para determinar o tempo óptimo da lise, de modo a que a libertação de fagos seja produtiva para a sobrevivência do fago. Para além destas duas proteínas essenciais, têm sido identificados novos genes nas cassetes de lise de bacteriófagos que infectam bactérias Gram-negativa. Estes genes, exemplificados pelas proteínas Rz e Rz1 codificadas pelo bacteriófago λ, comprometem a estabilidade das proteínas da membrana externa, eliminando a última barreira para a libertação dos viriões produzidos. Até à data nunca foram identificados homólogos destes genes em micobacteriófagos. A cassete lítica do micobacteriófago Ms6, fago que infecta *Mycobacterium smegmatis*, para além de codificar as duas proteínas essenciais, compreende três proteínas acessórias à lise codificadas pelos genes *gp1*, *gp3* (*lys*B) e *gp5*, restritos aos micobacterófagos. Apesar de serem classificadas com bactérias Gram-positivas, as micobactérias apresentam uma parede celular complexa. Esta parede celular consiste num peptidoglicano ligado covalentemente ao arabinogalactano, que por sua vez se encontra esterificado a uma variedade de ácidos gordos de cadeia longa (ácidos micólicos).

A presente dissertação caracteriza a proteína codificada pelo gene *lys*B do micobacteriófago Ms6 e o seu papel durante a lise. A análise bioinformática revelou a presença de um motivo conservado (GYSQG), característico de enzimas com actividade lipolítica. Usando a sequência de aminoácidos de LysB, foi realizada uma procura de similaridades nas bases de dados, através do programa BLAST, que identificou um grande número de proteínas similares. Após produção de uma proteína recombinante, His_6-LysB, em *E. coli* foi possível testar a sua actividade em diferentes substratos de enzimas lipolíticas. Os resultados demonstraram que LysB apresenta uma maior afinidade para substratos de cadeia longa (C_{16} e C_{18}). Foi possível demonstrar que o alvo de Ms6 LysB é a membrana externa de *Mycobacterium smegamtis,* ao clivar a ligação ester que liga os ácidos micólicos ao arabinogalactano no complexo micolil-arabinogalactano peptidoglicano. No entanto este substracto não é único uma vez que LysB também é capaz de hidrolisar o 6,6'-trealose dimicolato (TDM) de diferentes espécies de micobactérias. A construção de um micobacteriófago delecionado no gene *lys*B, demonstrou que o produto deste gene não é essencial

para a viabilidade do fago, mas participa no mecanismo de lise. Tendo em conta a complexidade do envelope das micobactérias, é fácil entender a necessidade dos micobacteriófagos adquirirem, durante a sua evolução, genes que lhes conferem uma vantagem evolutiva sobre aqueles que não os adquiriram de modo a conseguirem atingir uma lise mais rápida e eficiente.

Palavras Chave: Micobacteriófago Ms6, lisinas fágicas, enzimas lipolíticas; ácidos micólicos

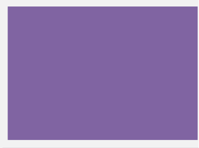


ABBREVIATIONS

aa	amino acid
AIDS	Acquired immune deficiency syndrome
AG	arabinogalactan
Amp	ampicillin
ATCC	American Type Culture Collection
ATP	adenosine-5'-triphosphate
BCG	bacille Calmette-Guérin
BLAST	basic local alignment search tool
bp	base pair
BRED	bacteriophage recombineering of electroporated DNA
BS	burst size
BSA	bovine serum albumin
CM	cytoplasmic membrane
CMN	Corynebacterium-Mycobacterium-Nocardia
Cryo-EM	cryo-electron microscopy
CWBD	cell wall binding domain
DADA-PCR	deletion amplification detection assay-PCR
DAT	di-acyl trehaloses
DEPC	diethylprocarbonate
DIM	phthiocerol dimycocerosates
DNA	deoxyribonucleic acid
DNP	dinitrophenol
ds	double-stranded
EDTA	Ethylenediamine tetraacetic acid
Fig	figure
FP	flanking primer
GC-MS	Gas-chromatography mass spectrometry
GlcNac	*N*-acetil glucosamine
gp1	mycobacteriophage Ms6 Gp1 gene
gp5	mycobacteriophage Ms6 Gp5 gene
GPL	glycopeptidolipis
HCl	hydroxide chloride
hol	mycobacteriophage Ms6 holin gene
ICTV	International Committee on Taxonomy of Viruses
IM	inner membrane

IPTG	isopropyl β-D-1-thiogalactopyranoside
Kan	kanamycin
lacZ	β-galactosidase gene
LAM	Lipoarabinomannan
LB	Luria-Bertani broth
LC-MS	Liquid chromatography-mass spectrometry
LIN	lysis inhibition
LM	lipomannan
Lpp	lipoprotein
LPS	lipopolysaccharide
lysA	mycobacteriophage Ms6 endolysin gene
lysB	mycobacteriophage Ms6 mycolylarabinogalactan esterase gene
MA	mycolic acids
mAGP	mycolyl-arabinogalactan peptidoglycan
MAMEs	mycolic acids methyl esters
m-DAP	meso-diaminopimelic acid
MDR-TB	multidrug-resistant tuberculosis
m.o.i.	multiplicity of infection
MraY	phospho-*N*-acetylmuramoyl-pentapeptide transferase
mRNA	messenger ribonucleic acid
Mtb TDM	trehalose 6, 6'-dimycolate from *M. tuberculosis*
MurA	UDP-*N*-acetylglucosamine-enolpyruvyl transferase
MurNac	*N*-acetylmuramic
NAM-amidase	N-acetylmuramoyl-L-alanine amidase
Ni-NTA	nickel-nitrilotriacetic acid
NCTC	National Collection of Type Cultures
OADC	oleic acid albumin dextrose complex
OD	optical density
OM	outer membrane
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
p.f.u.	plaque forming units
PG	peptidoglycan
PGL	phenolglycolipids
PGRP	peptidoglycan recognition protein
PIM	fosfatidilmio-inositol manoside
p.m.f.	proton-motive force
PMSF	phenylmethanesulfonylfluoride
pNP	*p*-nitrophenyl
pNPB	*p*-nitrophenyl butyrate
pNPC	*p*-nitrophenyl caprylate
pNPL	*p*-nitrophenyl laurate
pNPM	*p*-nitrophenyl myristate
pNPP	*p*-nitrophenyl palmitate
pNPS	*p*-nitrophenyl stearate
RBS	ribosome-binding site
RNA	ribonucleic acid
SAR	signal-arrest-release
SD	Shine-Dalgarno

SDS	sodium dodecyl sulphate
SL	Sulfolipids
SP	signal peptide
ss	single-stranded
TAT	tri-acyl trehaloses
TAG	triacylglycerols
TB	tuberculosis
TBAH	tetrabutylammonium hydroxide
TDM	trehalose 6, 6'-dimycolate
TLC	Thin-layer chromatography
TMD	transmembrane domain
TMM	trehalose monomycolate
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid
XDR	extensively drug-resistant
wt	wild type



PUBLICATIONS

The present work was mostly based on work that has been published, or is in preparation for publication, in international peer-reviewed journals:

Gil, F., Catalão, M. J., Moniz-Pereira, J., Leandro, P., McNeil, M. & Pimentel, M. 2008. The lytic cassette of mycobacteriophage Ms6 encodes an enzyme with lipolytic activity. *Microbiology* 154:1364-1371

Gil, F., Grzegorzewicz, A. E., Catalão, M. J., Vital, J., McNeil, M. R. & Pimentel, M. 2010. Mycobacteriophage Ms6 LysB specifically targets the outer membrane of *Mycobacterium smegmatis*. *Microbiology* 156:1497-1504.

Gil, F., Catalão, M. J., Milho, C. & Pimentel, M. The role of LysB in Ms6 infection cycle. (*manuscript in preparation*).

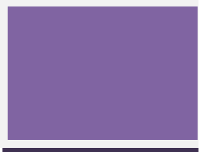
The following manuscripts have also been published during the Ph.D. studies:

Catalão, M. J., **Gil, F.**, Moniz-Pereira, J. & Pimentel, M. 2011. Endolysin-Binding domain encompasses the N-terminal region of the Mycobacteriophage Ms6 Gp1 Chaperone. *J Bacteriol* 193: 5002-5006.

Catalão, M. J., Milho, C., **Gil, F.**, Moniz-Pereira, J. & Pimentel, M. 2011. A second endolysin gene is fully embedded in-frame with the *lys*A gene of mycobacteriophage Ms6. *PLoS One* 6: e20515.

Catalão, M. J., **Gil, F.**, Moniz-Pereira, J. & Pimentel, M. 2011. Functional analysis of the holin-like proteins of mycobacteriophage Ms6. *J Bacteriol* 193: 2793-2803.

Catalão, M. J., **Gil, F.**, Moniz-Pereira, J. & Pimentel, M. 2010. The mycobacteriophage Ms6 encodes a chaperone-like protein involved in the endolysin delivery to the peptidoglycan. *Mol Microbiol* 77: 672-686.



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1

GENERAL INTRODUCTION

1. The *Mycobacterium* genera

The *Mycobacterium* genera include more than 100 species and belong to the Mycobacteriaceae family, order Actinomycetales, a large group of Gram-positive bacteria containing GC-rich DNA. Within this group, mycobacteria belong to one branch, called the *Corynebacterium-Mycobacterium*-Nocardia (CMN) branch. Mycobacteria can be distinguished between fast growing (generation time 3-5h) and slow growing (generation time 18-24h) species. Most of the species are saprophytic soil bacteria and a minority, belonging to the *Mycobacterium. tuberculosis* complex, is pathogenic to humans, causing old known diseases such as tuberculosis and leprosy (Jarlier & Nikaido, 1994). Tuberculosis (TB), also known as the white plague, is caused by the slow growing species *Mycobacterium tuberculosis*. It received the title of “captain of all these men of death” by John Bunyan in the second half of the XVII century, when the disease reached a high level of death rates in Europe. This disease became the principal cause of death by the end of the XIX and beginning of the XX century, and among its various victims were worldwide known people, such as Frédéric Chopin, Paganini, St. Francis of Assisi, Lord Byron and Elenor Roosevelt (Ducati *et al.,* 2006). Currently, this disease still represents a global threat, as it stands as the leading cause of death due to an infectious agent among adults worldwide with 1.1 million deaths and an estimated 8.9 million incident cases in 2010 (WHO Report 2011). In 2010, the Portuguese Health Authorities reported a TB incidence of 22 cases per 100,000 people nationwide (Antunes, 2010), being the European country with the higher incidence rate. The problematic of tuberculosis is worsened by the increasing incidence of MDR (multiple drug-resistant) tuberculosis, defined as strains that are resistant to isoniazid and rifampicin (Telenti & Iseman 2000), and more recently XDR (extensively drug-resistant) which means that these strains in addition to being resistant to isoniazid and rifampicin, are also resistant to any member of the quinolone family and at least to one of the second-line anti-tuberculosis injectable drugs (CDC 2006). Leprosy, a chronic disease with an estimated 250.000 new cases annually is cause by *M. leprae* (Koremromp *et al.,* 2009). Nontuberculosis mycobacteria, like the M*ycobacterium avium* complex, *Mycobacterium kansasii*, *Mycobacterium fortuitum*, and *Mycobacterium chelonae*, cause severe disease in immunodeficient individuals, such as those with AIDS or cystic fibrosis however, disseminated infections can also appear in healthy individuals (Al-Muhsen & Casnova 2008).

One of the major differences of mycobacteria is their unique cell wall structure (Fig. 1). The chemical nature of the mycobacteria envelope is different from those of Gram-negative and

Gram-positive bacteria, with a lipid content that may represent up to 60% (Neyrolles & Guilhot 2011) of the cell dry mass, compared to 5-10% in Gram-positive and Gram-negative bacteria (Goren & Brennan 1979). This high lipid content accounts for a high hydrophobicity that contributes to the low permeability to nutrients and antibacterial drugs, which slows down the growth of mycobacteria and makes disease caused by pathogenic species difficult to treat (Jarlier & Nikaido 1990). Another distinguishing property shared among mycobacteria is the fact that their cell wall retains carbol fuchsin dye even in the presence of acidic alcohol, for this reason mycobacteria are also known as acid fast bacilli (Glickman & Jacobs 2001).

1.1. The mycobacteria envelope

The envelope of mycobacteria consists of the cytoplasmic membrane surrounded by the cell wall core which is composed of peptidoglycan (PG) covalently attached to arabinogalactan (AG), which is, in turn is attached to the mycolic acids forming the mycolyl arabinogalactan-peptidoglycan (mAGP) complex (Brennan, 2003). These covalently linked mycolic acids comprise all or part of the inner layer of a true outer membrane (Hoffman *et al.,* 2008; Zuber *et al.,* 2008). Finally, outside of the outer membrane is a layer of protein and polysaccharides known as the capsule (Lemassu & Daffé 1994; Lemassu *et al.,* 1996). The plasma membrane is structurally and functionally similar to other bacterial cytoplasmic membranes (Silva & Macedo 1983; Daffe *et al.,* 1989), where polar lipids, mainly phospholipid, assemble themselves, in association with proteins, into a lipid bilayer (Ortalo-Magné *et al.,* 1996b) and are almost invariably derivatives of phosphatidic acid. The most common phospholipids of the cytoplasmic membrane are the phosphatidylinositol mannosides (PIM), phosphatidylglycerol, cardiolipin and, phosphatidylethanolamine (Ortalo-Magné *et al*., 1996b). Other components associated with the cytoplasmic membrane include a number of polyterpene-based products thought to be associated with protection against photolytic damage, such as the carotenoids, and the menaquinones that are involved in electron transport (Brennan, 1988).

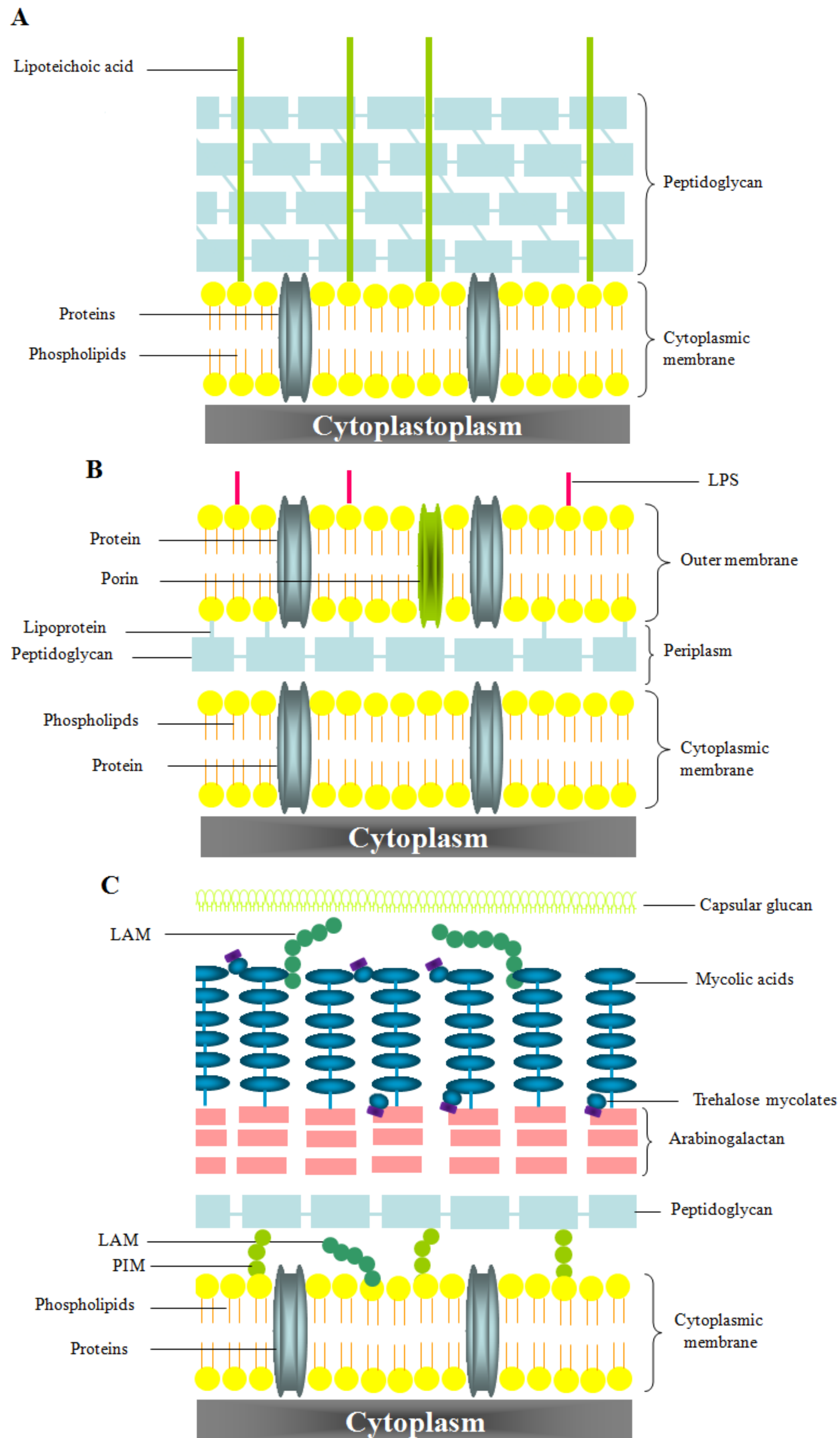
A
Lipoteichoic acid
Peptidoglycan
Proteins
Phospholipids
Cytoplasmic membrane
Cytoplastoplasm
B
LPS
Protein
Porin
Outer membrane
Lipoprotein
Peptidoglycan
Periplasm
Phospholipds
Protein
Cytoplasmic membrane
Cytoplasm
C
Capsular glucan
LAM
Mycolic acids
Trehalose mycolates
Arabinogalactan
Peptidoglycan
LAM
PIM
Phospholipids
Proteins
Cytoplasmic membrane
Cytoplasm

Figure 1. Cell envelopes of bacteria. A) Gram-positive bacteria; B) Gram-negative bacteria; C) Mycobacteria. Abbreviations: PIM, fosfatidil*mio*-inositol manoside; LPS, Lipopolissacaride. A) and B) adapted from Nikaido *et al.,* 1994; C) adapted from Sherman, M. (unpublished work).

The carotenoids of mycobacterial are responsible for the characteristic yellow-orange color of photochromogenic mycobacterial such as *M. gordonae* and *M. kansasii* (Brennan & Nikaido 1995).

Although mycobacteria are classified as Gram-positive bacteria, the existence of a compartment analogous to the periplasmic space in Gram-negative bacteria has been proposed by Daffé & Drapper (1998). Recent advances in deciphering the structure of the mycobacteria cell envelope confirm the existence of a periplasmic space in (Hoffman *et al.,* 2008; Zuber *et al.,* 2008; Sani *et al.,* 2010).

The peptidoglycan consists of alternating units of N-acetylglucosamine and N-glycolylmuramic acid cross-linked by tetrapeptides chains (Besra & Brennan 1997). The structure of mycobacterial peptidoglycan is slightly different from that of the common type. Many of the muramic acid residues are N-glycolylated with a glycolyl residue rather than the usual acetyl residue (Uchida & Aida 1979). In mycobacteria, except in *M. leprae*, the peptide chain consists of L-alanine-D-isoglutamic acid-diaminopimelic acid-D-alanine and the diaminopimelic acid is amidated (Mahapatra *et al.,* 2005). The amino acids cross-link is between residues of diaminopimelic acid or between diaminopimelic acid and D-alanine (Azuma *et al.,* 1970). The peptidoglycan is covalently bound to arabinogalactan, a complex branched heteropolysaccharide that contains a galactan chain composed of altering 5- and 6-linked D-galactofuranosyl residues (McNeil & Brennan*,* 1991) to which two arabinan chains of 20-31 residues each are attached (Bhamidi *et al.,* 2011). The terminal pentaarabinoside is in turn esterified by long chain (C_{60}-C_{90}) α-alky, β-hydroxy fatty acids, the mycolic acids (Brennan & Nikaido 1995; Bhamidi *et al.,* 2011). Two families of mycolic acids are known: α mycolates without any oxygenated functional groups and the oxygenated mycolates. Slow-growing pathogenic mycobacteria such as *M. tuberculosis* modify their mycolic acids by introducing cyclopropane rings (cyclopropanation), whereas fast-growing saprophyte species such as *M. smegmatis* do not (Chatterjee, 1997). These fatty acids constitute a significant proportion of the mass of the cell wall core and form a hydrophobic permeability barrier that surrounds the bacterium through hydrophobic interaction (Minnikin 1982; Brennan & Nikaido 1995). This feature is responsible for part of the endogenous resistance of *M. tuberculosis* to many drugs (Jarlier & Nikaido 1994; Drapper 1998). It has recently been shown that mycolic acids fold in a 4 column “W”

confirmation both by physical methods (Villeneuve *et al.,* 2005; Villeneuve *et al.,* 2007) and by the dimension of the outer membrane (Hoffman *et al.,* 2008; Zuber *et al.,* 2008).

The existence of this outer membrane bilayer has long been postulated (Minnikin*,* 1982) but only recently has been demonstrated in various mycobacterial species, through the direct visualization of vitreous sections under a cryo-electron microscope (Hoffmann *et al.,* 2008; Zuber *et al.,* 2008).

Embedded somehow in the cell wall core is a variety of extractable of lipids, like the glycolipids that are synthesized by all cultivable mycobacterial species, such as trehalose monomycolate (TMM), trehalose dimycolates (TDM), while others like sulfolipids (SL), phenolglycolipids (PGL), di- and tri-acyl trehaloses (DAT and TAT), glycopeptidolipis (GPL) and phthiocerol dimycocerosates (DIM) (Asselineau, 1998), are limited to specific species. Many of these are suspected to be present in the outer leaflet of the outer membrane and biosynthetically it is logical that TMD and TDM are present in the inner leaflet of the outer membrane as well as being present in the outer leaflet as these two glycolipids are the source of the mycolic acids attached to the arabinosyl residues of the cell wall core.

Also, interspersed somehow are the cell wall proteins, and the lipid anchored polysaccharides such as lipomannan (LM) and lipoarabinomannan (LAM). LAM is exposed on the surface and directly implicated in the immunopathogenesis of leprosy and tuberculosis (Brennan *et al.,* 1990). Thus LAM and LM may be anchored in the plasma membrane or the outer membrane or both; the available information is ambiguous on their location (Ortalo-Magné *et al.,* 1996a). The phosphatidylinositol manosides (PIMs which are essentially the lipid anchor regions of LM and LAM with only minimal additional glycosylation) have long been thought to be present in the plasma membrane but may also be present in the outer membrane. Finally, the phthiocerol-containing lipids, which lack a polar head group are known to be exported from the inside of the cell (Cox *et al.,* 1999) but the extracellular location is to be known. In mycobacteria, the presence of a low number of pore forming proteins relatively to *Escherichia coli* may explain the low permeability and the low susceptibility to toxic agents (Niederweis, 2003).

The occurrence of a capsule surrounding this outer membrane, has now been well established by Ortalo-Magné *et al.* (1995) who first analyzed the composition of the putative capsule established that the main components were polysaccharides and proteins, with much smaller amount of lipid. In 2010, Sani *et al.* demonstrated the presence of a capsule in both pathogenic

and non-pathogenic mycobacteria (including *M. smegmatis*, *M. marinum* and *M. tuberculosis*). In *M. marinum* this capsular layer, in addition to containing arabinogalactan, glycan and mannose-containing glyco-lipids, also surprisingly contains a large amount of ESX-1-secreted proteins.

More recently, there has been an urgent need for new antituberculosis drugs to combat the high incidence of drug resistant disease. Bacterial envelopes are, in general, essential for survival under normal conditions. Since mycobacteria envelopes contain many unique structures, they are obvious targets for novel drugs, and this led to renewed interest in structures and biosynthetic pathways.

2. Bacteriophages

Bacteriophages constitute a group of viruses that specifically infect bacteria. They are known to attack over 150 bacterial genera (Ackerman, 2009). Bacteriophages, as bacteria, are very common in all natural environments and it is estimated that there are about 10^{31} phages on earth and approximately 5500 have been characterized and reported (Ackerman, 2009). Bacteriophages are universally observed in open and coastal waters, marine sediments, terrestrial ecosystems such as soil, and the bodies of humans, animals and insects (Ackerman, 2003). They are not only the most abundant biological entities but probably also the most diverse ones and are characterized by a high degree of mosaicism that likely arises from extensive horizontal genetic changes (Hendrix *et al.,* 1999; Hendrix, 2003, Hatfull, 2008). The majority of the sequence data obtained from phage communities has no equivalents in databases. The phage population is dynamic, turning over rapidly through constant attrition and subsequent amplification in permissive host (Pedulla *et al.,* 2003). According to the International Committee on Taxonomy of Virus, bacteriophages classification includes 1 order and 14 families and 37 genera (Fauquet *et al.,* 2005). Bacteriophages are composed of a protein shell, the capsid, often in the shape of icosahedrons. The capsid encloses the viral genome, which can be ssDNA (single-stranded DNA), dsDNA (double-stranded DNA), ssRNA (single-stranded RNA) or dsRNA (double-stranded RNA) with most known bacteriophages having dsDNA. The majority of phages carry a more or less complex tail to which a base plate, spikes, or tail fibers can be attached. These structures are involved in recognition and attachment to phage receptors present at the bacterial surface. It was noticed that 5280 out of the 5500 reported phages are tailed phages, which are composed of an icosahedral head and a tail (Ackerman, 2009). Tailed phages are dsDNA phages and belong to three families according to

the morphological features of the tail: *Myoviridae* (contractile tail), *Siphoviridae* (long noncontractile tail) being the most abundant family including 61% of the phages described, and *Podoviridae* (extremely short tail). The rest of the phages, constituting only 4% of the total, are classified into 11 families. They are cubic, filamentous, or pleomorphic phages with dsDNA, ssDNA, dsRNA, or single-stranded RNA (ssRNA) genome and some types contain lipids in their envelopes or internal constituents (Ackerman, 2009).

Except for filamentous phages, which continuously extrude from their hosts without causing host lysis, all other phages are lytic phages that encode gene products to compromise or destroy the bacterial cell wall, leading to bacteriolysis (Young, 2005). For phages that end their vegetative cycle by lysing their hosts, disruption of the host cell wall may be accomplished in two ways: i) tailed phages use the holin-endolysin strategy to hydrolyze the bacterial murein layer; ii) phage lacking tails synthesize a single lytic factor that compromises the strength of the cell wall (Young, 2005).

According to their type of life cycle, dsDNA phages can be classified into virulent or temperate (Fig. 2). Virulent phages follow a lytic cycle that ends with lysis and death of the host bacteria cell, whereas temperate phages may follow a lytic or a lysogenic cycle. Phage infection starts with adsorption to specific receptors on the bacterial cell and the host specificity of the phage is usually determined by the type of tail fibers that a phage has. The nature of the bacterial receptor varies for different bacteria. Examples include proteins on the outer surface of the bacterium, teichoic acids, lipopolysaccharides, polysaccharides and lipoprotein (Rakhuba *et al.*, 2010). Adsorption is followed by transfer of phage genome into the host cell. The viral genome is generally transcribed by host cell RNA polymerase, producing early mRNA that has the effect of taking over the metabolic machinery of the bacterium, redirecting its metabolic processes to the manufacture of new virus components. These components are then assembled into complete virions. Once the new phage particles are formed, cell lysis occurs in order to liberate the new virions, which are now able to infect neighbor host (Young *et al.,* 2000).

In the lysogenic cycle the phage DNA may integrate into the host genome and spend part of their life cycle in a quiescent state called prophage but may also exist as a plasmid. Prophage DNA will be replicated when the host cell genome replicates and so daughter cells will inherit the viral DNA. Bacterial cells containing prophages – lysogenic cells – may undergo several rounds of division but occasionally one will spontaneously lyse and liberate progeny phage.

Alternatively, a population of lysogenic cells may be induced to lyse by subjecting them to stress, such as treatment with mutagenic agents or exposure to ultraviolet light, and undergo a lytic cycle with the release of phage progeny (Ranquet *et al.,* 2005).

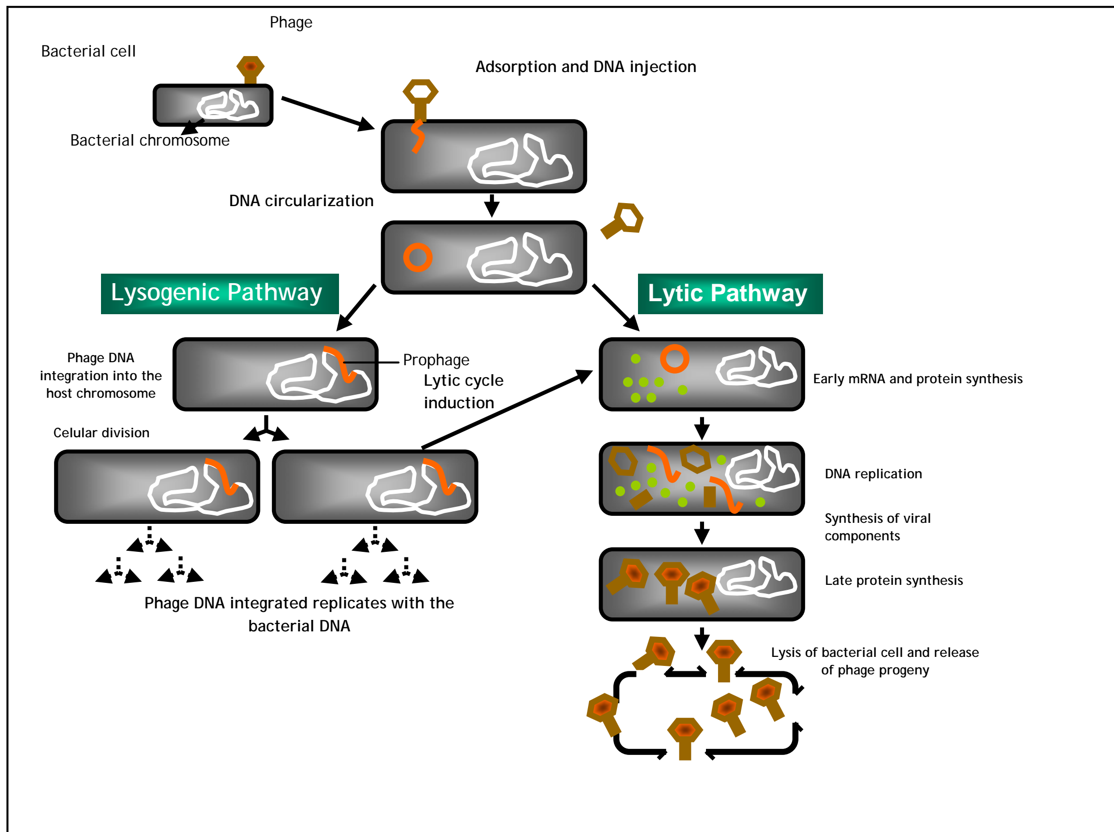

Figure 2. Lytic and lysogenic cycles exemplified by bacteriophage λ. See text for more complete description. Figure adapted from Hanlon, 2007.

3. Strategies for host lysis

As mentioned above, the final stage of a phage vegetative cycle is the release of newly synthesized phage particles. This is an important step regarding phage survival and ecological fitness. A sharply time-defined and efficient release of phage progeny is crucial to maximize both the burst-size (number of phages that are produced per phage-infected bacterium) and the opportunity to infect new hosts (São José *et al.,* 2007). The main barrier of host lysis is the continuous meshwork of peptidoglycan so compromising the cell wall is thus the fundamental goal for lytic processes (Young *et al.,* 2000).

3.1. Phages with single-stranded genomes

Unlike most of the well-studied phages, which have large dsDNA genomes, lysis strategy of small single-stranded genomes (ssDNA or ssRNA, fewer than 6kb) is poorly understood (Bernhardt *et al.,* 2002a). These phages developed a lysis mechanism with a single lytic protein (Young, 1992) probably because of the limited coding capacity of their small-size genomes. In these cases, the lysis activity appears to have evolved late, after the replicative and morphogenesis functions, because it is either a secondary activity of a morphogenesis protein or is encoded in a short alternate reading frame embedded in an essential morphogenetic cistron (Young, 2000). These lysis proteins act as "protein antibiotics", inhibiting cell wall synthesis and promoting septal catastrophe as the cell attempts division (Bernhardt *et al.,* 2002a). The only well characterized phages of this class are coliphages and among them there are three different prototypical lysis proteins: 1) the L protein of the ssRNA bacteriophage MS2 (*Leviviridae*), 2) the E protein of the single-stranded ssDNA bacteriophage φX174 (*Microviridae*), and 3) the A_2 protein of the ssRNA bacteriophage Qβ (*Alloleviviridae*) (Bernhardt *et al.,* 2002a).

Phage φX174 lysis gene, *E*, and phage Qβ lysis gene, A_2, encode proteins that specifically inhibit enzymes in the host murein synthesis pathway, while protein E inhibits the phospho-*N*-acetylmuramoyl-pentapeptide transferase (MraY) an integral membrane protein (Bernhardt *et al.,* 2001a; Mendel *et al.,* 2006), that catalyzes the synthesis of the first lipid-linked intermediate in peptidoglycan synthesis (Bernhardt *et al.,* 2000; Bernhardt *et al.,* 2001a), the Qβ A_2 inhibits the UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA), a soluble enzyme catalyzing the first step of murein precursor synthesis, respectively (Bernhardt *et al.,* 2001b). However, the function of the MS2 lysis protein, L, remains unknown. Since at least two out of three prototypical lysis proteins of small phages inhibit a specific step in peptidoglycan synthesis, this may be a general lysis strategy employed by many ssDNA and ssRNA phages of limited genome size. No muralytic enzyme activity has been detected in the lysates of these phages, and the analysis of their sequence does not revealed the presence of any muralytic enzyme. Based upon their activity, the term *amurin*, to indicate their negative effect on murein synthesis, has been proposed to identify this class of lysis proteins (Young 2002).

3.2. Phages with double-stranded DNA genomes

dsDNA phages encode two proteins that have independently evolved to accomplish both rapid progeny release and optimal lysis time. One of these proteins is an endolysin, a peptidoglycan hydrolase, which is a soluble, non-structural component of the virion. The other protein involved in lysis is the holin, which is responsible for defining the optimal timing for lysis and is also responsible for either the endolysin activation or the endolysin access to the peptidoglycan. Evolutionary speaking, time-defined lysis is advantageous to the phage and it is a trait under high selective pressure depending on host fitness and abundance (Wang *et al.,* 1996; Wang 2006).

3.3. Proteins involved in phage lysis

3.3.1. Endolysins

The name endolysin was coined in 1958 to designate a probably proteinaceous lytic substance synthesized in bacterial cells during phage multiplication and acting on the cell wall from inside the cell (Jacob & Fuerst, 1958). They are characterized by their ability to directly target bonds in the peptidoglycan (PG) of the bacterial cell wall, resulting in the degradation of the murein layer and release of newly assembled virions by way of lysis (Loessner, 2005). Endolysins are clearly distinguished from the lytic enzymes, since these enzymes are components of the virion and are able to digest the cell wall from the outside to allow the phage genome to be injected into the host cell.

Endolysins can be classified according to their catalytic activity as N-acetylmuramidases (lysozymes or muramidases), endo-β-N-acetylglucosaminidases (glucosaminidases), transglycosylases, N-acetylmuramoyl-L-alanine amidases (NAM-amidases), and endopeptidases. Glucosaminidases, lysozymes, and transglycosylases act on the sugar moiety (glycosidases) of the peptidoglycan whereas amidases hydrolyze the amide bond between the sugar of NAM and L-alanine residues in the oligopeptide crosslinking chains and endopeptidases cleave one of several different peptide linkages in the oligopeptide cross-links (Fig. 3) (Hermoso *et al.,* 2007). Endolysins usually possess only one type of hydrolytic activity, but some enzymes harboring two independent activities have already been reported. Examples are the endolysins of phages B30 (Pritchard *et al.,* 2004) and NCTC 11262 (Cheng *et al.,* 2005) both infecting *Streptococcus agalactiae* (endopeptidase and Lysozyme), *Staphylococcus aureus* phage φ11 (endopeptidase and NAM-amidase) (Navarre *et al.,* 1999) and the prophage φWMY from *Staphylococcus warneri* M (endopeptidase and NAM-amidase) (Yokoi *et al.,* 2005).

Many endolysins, especially from phages infecting Gram-positive host have a modular structure, with a catalytic domain located at the N-terminal, and a cell wall binding domain (CWBD) located at the C-terminal end, which may bind a species-specific carbohydrate epitope in the cell wall (Hermoso *et al.,* 2007).

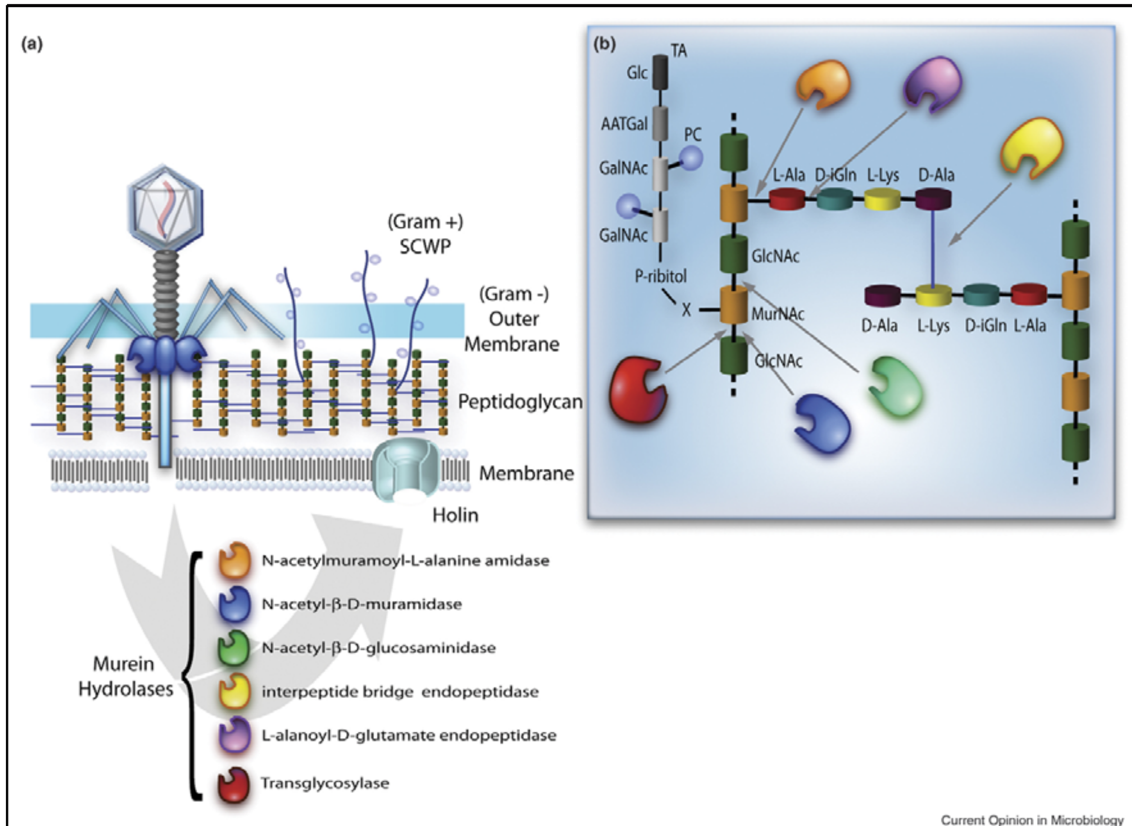

Figure 3. Bacterial cell wall structure and endolysin targets. (a) Schematic representation of the bacterial cell wall; (b) peptidoglycan structure (gram-positive bacteria) and endolysins targets. Figure from Hermoso *et al.,* 2007.

Most endolysins are not endowed with N-terminal secretion signals that would allow them to cross the cytoplasmic membrane, and are thus dependent on holin function to access their target. However, recent findings have shown that some endolysins are exported to the extra-cytoplasmic environment in a holin independent way. The first report came from the work of Parreira *et al.* (1999), on the *Oenococcus oeni* phage fog44 (Fig. 4). It was demonstrated that the endolysin Lys44 contains an N-terminal cleavable signal-peptide and that is continuously exported to the cell wall from the moment of its synthesis, involving the host *sec* translocon (São José *et al.,* 2000).

It was proposed that Lyz44 protein is kept in an inactive state in the murein layer, perhaps by local conditions that directly inhibit the catalytic site of the enzyme (São José *et al.,* 2000). Regardless the fact that the endolysin is secreted to the periplasm, fog44 apparently has a holin gene, *hol44* (Parreira *et al.,* 1999). A search of orthologous endolysins from phages infecting Gram-positive bacteria revealed that some of these endolysins had N-terminal sequences resembling secretory signals, and also in every case an adjacent holin gene is predicted. It is clear that in these phages the holin is not required for the export of the endolysin but its presence suggests that the lysis clock is regulated by holin-mediated membrane disruption that would somehow activate the exported endolysin rather than allowing their passage through the cytoplasmic membrane (São José *et al.,* 2000). Indeed, Nascimento *et al.* (2008) have shown that bacterial membrane proton-motive force regulates the lytic activity of the secreted endolysin Lys44. Experimental evidence for other secreted endolysins has been obtained for the lytic enzymes of phages φAM2 (São José *et al.,* 2000) and φg1e (Kakikawa *et al.,* 2002) from *Lactococcus lactis* and *Lactococcus plantarum*, respectively.

In addition to these endolysins endowed with a signal peptide, a new class of endolysins named SAR (signal-arrest-domain) endolysins was recently identified (Fig. 4). The first description of these endolysins results from the work of Xu *et al.,* (2004), on the endolysin of phage P1. The N-terminal of P1 Lyz differs significantly from the fOg44 secretory endolysin in that it does not have a secretory signal sequence. Instead its N-terminal domain presents a TMD, rich in residues that are weakly hydrophobic, functioning as a signal-arrest-release (SAR) sequence. The SAR domain engages the *sec* system directing the endolysin to the periplasm in an inactive membrane–tethered form, which is then activated by release from the membrane to a soluble state without proteolysis (Xu *et al*., 2004). Triggering of programmed lysis by the phage holin will allow a very rapid, and quantitatively significant, release of the SAR endolysin from the membrane which in turn, causes activation of the endolysin leading to a rapid lysis. The SAR domain of Lyz has two functions. First, it acts as a signal-arrest sequence and mediates the transport, and association, of the phage endolysin to the membrane. Once the protein is released, its C_{13} residue is involved in the isomerisation reaction that relieves topological, covalent, and conformational constraints from Lyz, which directs the protein to assume an active conformation mediated by DsbA (the periplasmic primary oxidant involved in disulfide bond formation), and possibly facilitated by periplasmic foldases and chaperones (Xu *et al.,* 2004; Xu *et al.,* 2005).

It was demonstrated that the exported P1 endolysin is kept inactive by three levels of control - topological, conformational, and covalent- until its release from the membrane is triggered by

the P1 holin (Xu *et al.,* 2005): this functional regulation is essential to avoid premature lysis of the infected host. More recently, the regulation of the muralytic enzyme of phage 21 revealed that endolysin R also possesses a SAR domain. Both genetic and crystallographic studies show that the SAR domain of R^{21}, once extracted from the bilayer, refolds into the body of the enzyme and effects muralytic activation by repositioning one residue of the canonical lysozyme catalytic triad: besides controlling the topology of protein, the SAR domain in R^{21} plays a specific and more integral role in the catalytic activity of the enzyme. Once synthesized, SAR endolysins require both strict post-secretory negative regulation and the means to become activated in a timely manner, because they are not sequestered from their substrate by the membrane. Inactive Lyz^{P1} has two levels of negative control — covalent inactivation of its active-site cysteine and an N-terminal catalytic domain with radical conformational disability — whereas R^{21} seems to lack only the correct placement of its catalytic glutamate (Sun *et al.,* 2009).

A sequence comparison has identified several endolysins from phages of Gram-negative bacteria having N-terminal sequences that resemble the SAR sequences. 57% of the residues in the SAR sequences of T4 lysozyme homologs are either weakly hydrophobic Gly and Ala, or uncharged polar residues, Ser, Thr, Gln, and Tyr, in contrast to only 36% in a transmembrane domain (TMD) (Xu *et al.,* 2004).

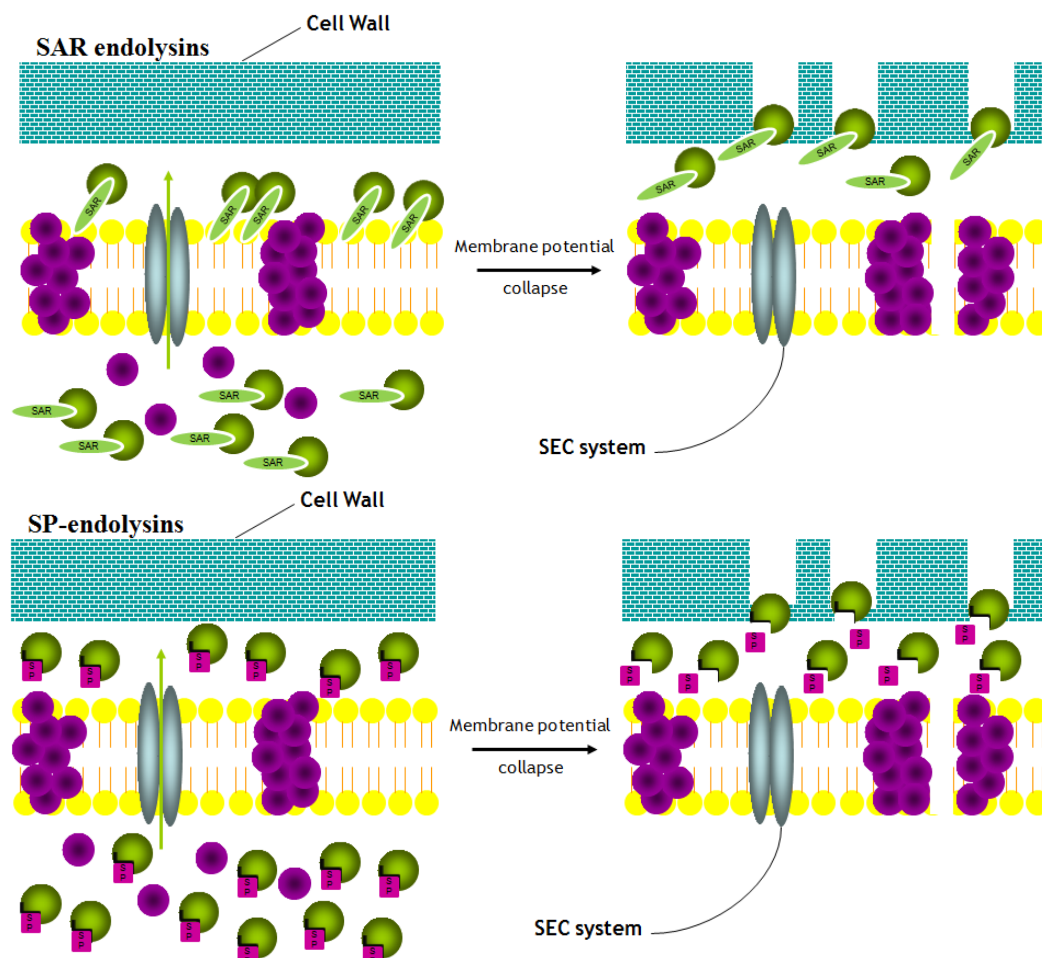

Figure 4. Model representation of host-lysis strategies of phages producing SAR endolysins or signal peptide-bearing endolysins. See text for further description. Figure adapted from São-José *et al.,* 2003.

3.3.2. Holins

Unlike endolysins, holins are much more diverse and frequently unique with respect to their primary sequence and they may be defined as small proteins (<150 aa) with at least one predicted TMDs, a hydrophilic, highly charged C-terminus and are frequently located immediately upstream of a readily identifiable endolysin gene (Wang *et al.,* 2000; Young 2002). The high frequency of this particular gene arrangement in phage infecting both Gram-positive and Gram-negative bacteria has allowed the tentative assignment of holin functions based on sequence analysis alone but predictions may be less straightforward however, if deviations in gene arrangement are observed (São José *et al*., 2003).

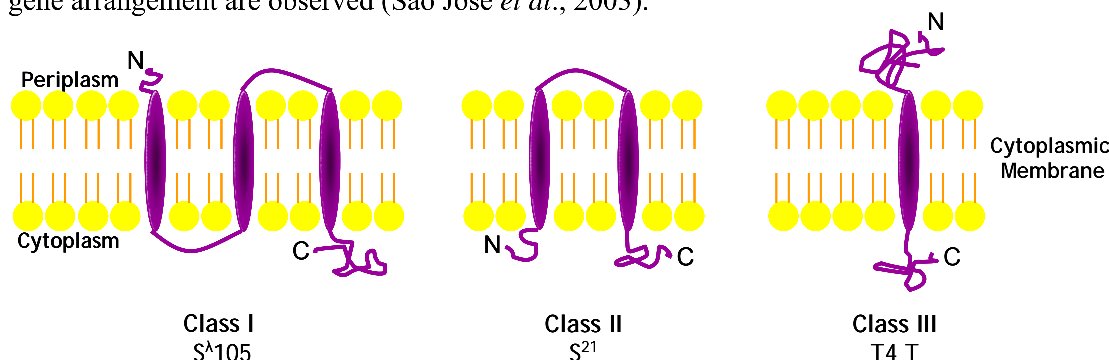

Figure 5. Schematic representation of known topologies of described phage holins. Adapted from São-José *et al.*, 2003.

Holins have been classified in three different classes I to III according to the predicted or experimentally determined number of TMDs, three, two or one, respectively (Wang *et al.,* 2000). The λ (class I) and T4 (class III) holins are cases where membrane topology has been experimentally determined, showing a N_{out}-C_{in} and N_{in}-C_{out} configuration, respectively (Fig. 5). For the holin of lambdoid phage 21 (S^{21}), the prototype of class II holins, it has been suggested a N_{in}-C_{in} topology (Bläsi & Young 1996; Barenboim *et al.,* 1999). However, some class II members may present alternative membrane topologies as suggested for the holin of the *Clostridium perfringens* phage φ3626 holin, for which topology prediction indicates that both N- and C-termini face the outside of the membrane (Zimmer *et al.,* 2002).

Holins are late synthesized proteins, that progressively accumulate in the cytoplasmic membrane of the host and while the proton-motive force is maintained, they assemble into oligomers and rafts of intrinsic stability (Young & Wang, 2006). Then, at a precise time programmed into its primary structure, they suddenly trigger to form a lesion that permeabilizes

the membrane. This event ends macromolecular synthesis and thus effectively terminates infection (Wang *et al.,* 2000; Young *et al.,* 2000). For canonical holins, the pores are large enough to allow the passage of the endolysins accumulated in the cytoplasm. Holins can be prematurely triggered by membrane depolarization with energy poisons such as cyanide and dinitrophenol (DNP) (Gründling *et al.,* 2001; Young, 2005). So, holin function is thus solely directed to the determination of the length of the infective cycle and to allow the endolysin access to the peptidoglycan, either by forming pores in the membrane large enough or either by activating the endolysins already positioned in the extracytoplasmic compartment. Holins are subjected to opposing evolutionary forces; on one hand, there is pressure to extend the vegetative cycle to allow continued accumulation virions at a linear rate; on the other hand, there is pressure to trigger lysis earlier to release progeny phage particles that can infect new hosts and potentially yield exponential increases in phage numbers (Wang *et al.,* 2000; Young *et al.,* 2000).

For phage encoding SAR endolysins, holins need only to depolarize the membrane in order to fulfil their role in controlling the timing of lysis. The formation of large membrane lesions like those resulting from λS (Wang *et al.,* 2000) would not be necessary. This raises the possibility that holins serving SAR endolysins may not function with canonical, soluble endolysins to affect host lysis. Indeed, unlike lesions formed by the λ holin, lesions formed by the phage 21 holin do not allow the passage of λ endolysin (Park *et al.,* 2007). The term ''pinholin'' has been proposed to differentiate the small-hole (pinhole) forming character of the phage 21 holin from the canonical holins that form large, nonspecific holes (Park *et al.,* 2007). This pinholin has two transmembrane domains, but only TMD2 is required for the pinhole (Park *et al.,* 2006). For both canonical holins and pinholins, hole formation occurs suddenly after a period of harmless accumulation in the membrane, during which time membrane energization, macromolecular synthesis and virion assembly continues undisturbed. Triggering determines the timing of lysis, although the molecular strategies are completely different for both (Pang *et al.,* 2009).

It was suggested that the S^{21}/R^{21} gene pair, encoding a pinholin and a SAR endolysin, may represent an intermediate stage in the evolution of holin-endolysin systems. The minimum requirement for an effective phage lysis system, other than the muralytic activity itself, is a delay in lysis after the onset of late phage expression, to allow assembly of progeny virions (Young & Wang, 2006). The most primitive dedicated lysis system could have consisted of a SAR endolysin alone. This mode would provide a lysis delay because of the gradual release and

activation of the membrane-tethered endolysins. However, a lysis system employing a SAR endolysin alone would be inherently inferior to canonical holin-endolysin systems for two reasons. First, because canonical holins function with cytoplasmic endolysins, the muralytic activity elaborated during the infection cycle can be produced in great excess. Not only does this mean that once the holin triggers, host lysis occurs in a matter of seconds, reducing the dwell time in the dead, non-productive host to a minimum, but also it means that lysis timing is completely dependent on the holin. Secondly, it has been shown that most missense changes in holin proteins alter the timing of lysis, unpredictably advancing or retarding the instant of triggering (Raab *et al.,* 1988; Johnson-Boaz *et al.,* 1994; Wang *et al.,* 2000, Rydman *et al.,* 2003). The canonical holins thus have a selective advantage not only for fitness, in terms of the mechanistic advantages of holin function, but also because they can function with either cytoplasmic endolysins or SAR endolysins, whereas the pinholin can function only with SAR endolysins (Park *et al.,* 2007).

Although holins appears to be the key factors determining timing of lysis, fine tuning of lysis regulation may involve phage proteins with a holin antagonistic function, the antiholins. In fact, Young & Bläsi (1995) argued that all phages employing a holin-endolysin system would also need an antiholin function as a mean to ensure the post-translational control of holin activity. In 20 out of 46 cases of phages infecting Gram-positive hosts in which a typical holin-lysin cassette was reported, a second holin-like gene, located immediately upstream of the *hol-lys* pair, could be detected (São-José *et al.,* 2003). Also, it cannot be excluded the situation where the holin functional unit is built from a complex of different polypeptides, rather than from a single protein. The *Bacillus subtilis* defective prophage PBSX has a late operon, identified as the lytic module, composed by the *xepA*, *xhlA*, *xhlB* and *xlyA* genes, and it was proposed that the two open reading frames preceding the endolysin *xlyA* (*xhlA* and *xhlB*) encode polypeptides that associate in the membrane to form a functional holin complex that allow XlyA access to the peptidoglycan (Longchamp *et al.,* 1994; Krogh *et al.,* 1998).

Many holin genes have potential dual-start motifs which would allow for the production of a shorter holin and a longer antiholin. In fact antiholins have been found to have widely different topologies and molecular features:

- On phage λ, the gene encoding the S holin has a dual-start motif which allows the production of the holin effector (S_{105}), produced from translation initiation at codon 3, and a holin inhibitor (S_{107} antiholin) resulting from translation initiation at codon 1 (Bläsi *et al.,* 1989, Bläsi *et al.,* 1990). S_{105} is a 105-residue integral cytoplasmic membrane protein with three TMD topology (N_{out}, C_{in}) whereas S_{107} share the same 105 aa sequence but has an

extra Met and Lys residues in the N-terminus that confers two extra positive charges. These extra charges prevent the translocation of the first TMD into an energized-membrane (Grundling *et al.,* 2000). S_{105} and S_{107} are produced in a 2:1 ratio approximately and the triggering time of lysis is inversely related to the excess of S_{105} over S_{107}. When S_{107} is made in excess of S_{105}, lysis does not occur in a physiological meaningful time frame (Raab *et al.,* 1988; Bläsi *et al.,* 1990; Chang *et al.,* 1995). When triggering occurs the first hole forms, the membranes depolarizes, allowing the N-terminal TMD1 of S_{107} in each S_{105}-S_{107} heterodimer to flip across the membrane; the inactive heterodimers are converted into active hole-forming subunits, thus instantly tripling the amount of active holin protein involved in compromising the membrane (Young, 2002).

- The P2 lysis cassette spans the genes *Y*, *K*, *lysA*, *lysB* and *lysC*, with Y encoding a class I holin and K encoding an ortholog of the R^{λ} endolysin. LysA appears to be an integral membrane protein with four TMDs and although non-essential, this protein affect the correct timing of lysis as *lysA* amber mutants causes early lysis and was designated as an antiholin. The remaining genes encode functional analogues of λ Rz/Rz1 (Ziermann *et al.,* 1994).
- T4-infected cells are subjected to "lysis inhibition", or LIN (Doermann 1948). The LIN state, in which the normal lysis timing of the holin is overridden, is established if a T4-infected cell undergoes superinfection by another T4 particle. In T4 phage the antiholin RI is predicted to be either a type II signal anchor protein with an N-terminal TMD, or a processed, secreted periplasmic protein (Young, 2005). Superinfection provides a signal that causes RI to inhibit T (holin) (Paddison *et al.,* 1998; Tran *et al.,* 2005) and somehow shifts the steady state of *RI* synthesis and degradation in favour of accumulation, allowing the formation of RI-T inhibition complexes (Ramanculov & Young, 2001).
- The analysis of P1 of *lyd* locus indicated that there are two genes, l*ydAB*, under the control of a late promoter, with *lydA* encoding a canonical class I holin and *lydB* encoding an antiholin. These genes are adjacent but unlinked to the endolysin gene, *lyz*. Unlike the case for both λ and T4, the antiholin LydB is essential to obtain a productive burst; in its absence, lysis occurs catastrophically early (Walker & Walker, 1980) indicating that LydB represses the P1 LydA holin function (Lobocka *et al.,* 2004).

There is clearly great diversity in the antiholin schemes evolved by bacteriophages. Presumably this reflects the evolutionary impact of achieving lysis ideally timed and consummated to suit every variation of the growth environment. Possibly it also reflects the easiness by which holin timing can be altered, perhaps by protein-mediated interaction with the holins that distort the fragile balance between maintenance of the pre-hole state and the formation of the catastrophic lytic lesion (Young, 2002).

3.3.3. Other proteins involved in lysis – Rz/Rz1 proteins

The lytic cassette of λ phage comprises the two genes described to be essential for lysis, gene *S* (holin) and the gene *R* (endolysin) and also two additional genes *Rz* and *Rz1* (Young, 1992).

λRz encodes a 153 amino acid polypeptide that is predicted to be a type II integral membrane protein, with an N-terminal transmembrane domain (TDM) and a periplasmic C-terminus (Summer *et al.,* 2007). The *Rz1* coding region is embedded entirely within *Rz* in the +1 reading frame (Fig. 6) and is predicted to encode a small (40 aa) proline-rich outer membrane lipoprotein lacking similarity to any protein of known function (Summer *et al.,* 2007). In the presence of milimolar concentrations of divalent cations, lysis is prevented if both Rz and Rz1 are inactivated, leading to the accumulation of spherical mechanically fragile cells (Young *et al.,*, 1979; Zhang and Young, 1999).

Berry *et al.* (2008) demonstrated that, at physiological levels of expression, Rz and Rz1 are localized to inner membrane (IM) and outer membrane (OM) respectively, with their C-terminal domains predicted to lie in the periplasm. Recently, it was demonstrated that these two proteins form a complex (Berry *et al.,* 2010) and it was proposed their involvement in a third and final step in host lysis. The first step is the temporally programmed permeabilization of the cytoplasmic membrane by the holin, resulting in the release of a cytoplasmic endolysin or the activation of a SAR endolysin. The second stage is the degradation of the peptidoglycan by the endolysin. These two stages are followed by a third stage involving the fusion of the IM and OM mediated by the Rz/Rz1 complexes. This would cause the cytoplasm and the environment to become topologically equivalent and, thus, all conceivable barriers to the release of the progeny virions would be eliminated. It was suggested that to facilitate the fusion of the two membranes, Rz/Rz1 complexes might undergo conformational changes (Jahn & Scheller, 2006). Although this final step is not required in typical laboratory medium lacking sufficient divalent cations to stabilize the OM, the lack of Rz or Rz1 function is noticeable as a diminution in the sharpness of lysis profiles, not only in lysis mediated by SAR endolysins but also with the canonical lambda *S/R* holin-endolysin lysis (Summer *et al.,* 2007).

A bioinformatics search identified Rz/Rz1 equivalents in nearly all genomes from phages that infect Gram-negative bacteria (Summer *et al.,* 2007). The variety of Rz/Rz1 equivalents was enormous with 37 unrelated gene families grouped in accordance to gene arrangement. In the lambda group Rz/Rz1 pairs share the embedded gene architecture (8 families); other the P2 group, includes families in which Rz1 extends beyond Rz (overlapped structure; 23 families); the third group includes families (6 families) with Rz/Rz1 homolog's encoded in separated genes, as exemplified by the T4 pseT.3 and pseT.2 genes, that encode proteins unrelated to all other Rz/Rz1 equivalents but that possess the signature N-terminal TMD and outer membrane lipoprotein signal sequence respectively (Summer *et al.*, 2007). In the study performed by Summer *et al.,* (2007), functional homologous of RZ/RZ1 encoded by a single gene were also identified in seven phages, including the coliphage T1. The T1 gp11 and the Rz/Rz1 proteins are functionally equivalent. This new class of proteins, named spanins are predicted outer membrane lipoproteins that also possess a predicted C-terminal TMD and thus will span the entire periplasm, providing a physical link between the inner and outer membranes.

The fact that genes for Rz/Rz1 equivalents are found in nearly all phages of Gram-negative hosts indicates that in the osmotic and ionic conditions found in nature the OM is a significant barrier even after holin-endolysin mediated destruction of the cell wall and that they confer an advantage in nature that is not apparent under laboratory conditions (Summer *et al.,* 2007).

While the three steps of phage lysis mediated by holins, endolysins and Rz/Rz1 complexes form a sequential pathway, in which holin function is required for endolysin function, which is in turn required for Rz/Rz1 function, they are mechanistically independent (i.e. do not require heterotypic interactions with each other). This likely accounts for the remarkable diversity and mosaicism found in phage lysis cassettes, which are composed of many unrelated families of holins and Rz/Rz1 proteins, and at least three types of endolysins (Wang *et al.,* 2000; Young, 2002; Young and Wang, 2006; Summer *et al.*, 2007).

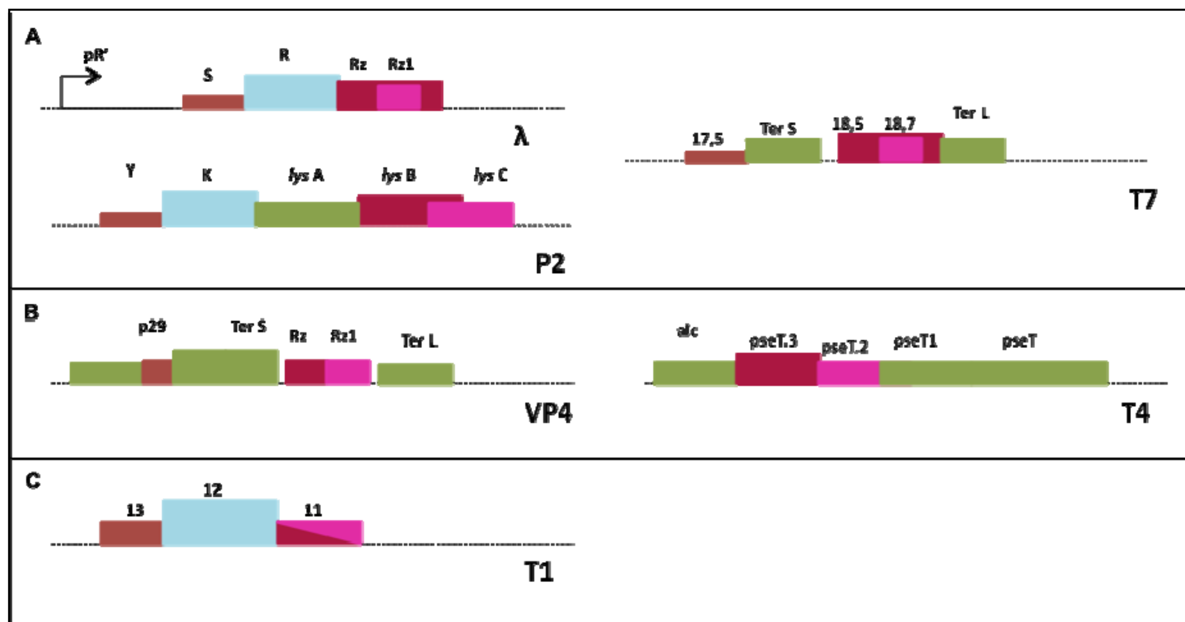

Figure 6. Rz-Rz1 and spanin gene arrangements. In each map, brown, light blue, red and pink boxes indicate holin, endolysin, Rz and Rz1 genes, respectively. (A) The prototype embedded (λ, T7), or overlapped (P2) Rz/Rz1 genes. (B) Separated Rz and Rz1 genes (VP4 and T4). For phage T4, the start codon for the Rz1 (pseT.2) gene overlaps the stop codon of the Rz (pseT.3) gene, although the protein coding sequences are separated. (C) The T1 spanin gene, shown as a box with half black and half white filling. Figure adapted from Summer *et al.,* 2007.

4. Phages that infect mycobacteria – Mycobacteriophages

Mycobacteriophages are viruses that infect mycobacterial hosts. Interest in mycobacteriophages began in the late 1940s with the isolation of phages that infect *Mycobacterium smegmatis* (Gardner *et al.,* 1947; Whittaker, 1950), followed by phages that infect *Mycobacterium tuberculosis* (Froman *et al.,* 1954). A primary motivation of these early studies was to type mycobacterial clinical isolates, which was further advanced by collecting sizable numbers of mycobacteriophages from a variety of environmental and clinical sources (Grange, 1975; Jones, 1975; Snider *et al.,* 1984). The major interest of these studies was in large part from the medical significance and biological idiosyncrasies of their hosts. Some phages like D29, have broad host ranges and infect many species of both fast-growing and slowly growing mycobacterial, including *M. smegmatis* and *M. tuberculosis* (Rybniker *et al.,* 2006), whereas others, like DS-6A have very narrow preferences and infect only bacteria of the *M. tuberculosis* complex (Redmond & Cater 1960; Rybniker *et al.,* 2006). Several phages discriminate between strains or isolates of particular species, and was noted that phage 33D differentiates between BCG strains and *Mycobacterium bovis* (Hatfull, 2010).

Realization of the full potential of mycobacteriophages contributes to an understanding of their hosts but it requires the characterization of their genomes; the first sequenced genome was that of mycobacteriophages L5 in 1993 (Hatfull *et al.,* 1993), followed by D29 and TM4 in 1998 (Doke, 1960; Donnelly *et al.,* 1993) and Bxb1 in 2000 (Jacobs *et al.,* 1987). Currently, more than 1390 mycobacteriophages were already isolated, most of all having *M. smegmatis* as host. The recent advance in the characterization of mycobacteriophages is mainly due to the work of Dr. Graham Hatfull, who develops a Phage Hunting program in the University of Pittsburgh contributing to the increase in the number of mycobacteriophages isolated. A comparative analysis of eighty one complete genome sequences was recently published (Hatfull *et al.,* 2010; Henry 2010). All the characterized mycobacteriophages are double-stranded DNA (dsDNA) tailed phages belonging to the order Caudovirales, restricted to two morphotypes, the *Siphoviridae* and the *Myoviridae* (Hatfull *et al.,* 2008).

4.1. Genomic diversity

The 81 sequenced mycobacteriophages genomes encompass substantial genetic diversity, and the genomic architectures are dominated by mosaic relationships. Any two particular phages may share either extensive nucleotide sequence similarity over the entire genome (e.g. phages Adjutor and PB11), or as few as three genes whose products share greater than 25% amino acid identity (e.g., phages Barnyard and Giles) (Hatfull, 2010). Based on the relationship between the genomes, mycobacteriophages were grouped in clusters. The criteria for clustering was that any two genomes with evident nucleotide sequence similarity spanning more than 50% of the genome lengths should de included within the same cluster. Sequenced mycobacteriophages were grouped into nine clusters named A through I and 5 mycobacteriophages have singleton genomes with no close relatives. The nine clusters were further subdivided into subclusters, and it was anticipated that as additional genomes are sequenced new clusters will be formed and that current clusters will undergo further subdivision (Hatfull *et al.,* 2011).

Hatfull *et al.* (2006) illustrated the mosaicism of mycobacteriophages genomics by grouping all the identified ORFs into “phamilies” of related sequences and then creating phamily circles based on their homology. Analysis of the full sequenced genomes allowed assembly of 9014 putative ORFs into phams, generating a total of 2345 phams, with 1108 phams containing only a single gene (orphams) (Hatfull *et al.,* 2011). It is noteworthy that about 15% of mycobacteriophages gene phamilies match non-mycobacteriophage sequences, suggesting that

new genes are also acquired from bacterial genomes during the course of their evolution (Hatfull *et al.,* 2006). Mycobacteriophage genome lengths vary greatly from 41,4 (Angel) to 164,6 kbp (Myrna) with an average length of 73,6 kbp. The considerable variation in genome organization can be demonstrated by mycobacteriophages with a siphovirial morphotype (all but Cluster C) since they all share a syntenic group of genes encoding virion structure and assembly proteins – as seen in all siphoviruses, regardless of their bacterial host and regardless of the lack of sequence similarity. Cluster C consists of mycobacteriophages with myoviral morphologies and relatively large genomes, and the virion structure and assembly genes do not appear to be organized into a well-defined array as they are in the siphoviruses (Hatfull, 2010). No Podoviridae phages have been described, probably due to the thick cell envelope of mycobacteria requiring a long tail to insert the DNA into the cytoplasm.

In summary, a detailed study of mycobacteriophage genes and protein prediction should contribute to a better understanding of the phage's mechanism of infection. This could bring new tools for the battle against mycobacterial diseases such as the production of recombinant anti-mycobacterial peptides based on the knowledge of key genomics regions such as the lytic enzyme cassette, as well as revealing interesting biological features of their unusual bacterial hosts (Henry, 2010).

4.2. Applications and Biotechnology

We have been assisting, all over the world, to an increase in bacteriophage studies regarding their potential as antimicrobial agents against pathogenic bacteria. While the full potential of mycobacteriophages has yet to be realized, several useful advances have been made. Mycobacteriophages have shown to be powerful tools to study mycobacteria genetics, by facilitating the development of mycobacterial genetic systems. Based on the integration systems of L5, FRATI and MS6, it was possible to construct integration-proficient plasmid vectors (REF). Other applications include methods for mycobacterial transformation (Hatfull, 2005), phage-based vectors (e. g., phasmids), non-antibiotic-selectable markers, gene expression systems, transposon delivery vehicles (Hatfull, 2000), gene replacement strategies using nonreplicating vectors (Husson *et al.,* 1990), long linear DNA fragments (Balasubramanian *et al.,* 1996), incompatible plasmids (Pashley *et al.,* 2003), counterselectable markers (Pelicic *et al.,* 1996), specialized transducing shuttle plasmids (Bardarov *et al.,* 2002).

More recently, recombineering systems for mycobacteriophages have been developed by Van Kessel *et al.,* (2007) and Marinelli *et al.,* (2008). The bacteriophage recombineering of electroporated DNA (BRED) provides for direct genetic manipulation of mycobacteriophages

that takes advantage of a mycobacterial-specific recombineering system (Van Kessel *et al.,* 2007; Marinelli *et al.,* 2008). This recombineering approach is based on the use of the RecET-like recombination system encoded by phages Che9c, such that expression of genes *60* and *61* generates high levels of recombination in both *M. smegmatis* and *M. tuberculosis* (Van Kessel *et al.,* 2007; Van Kessel *et al.,* 2008). The BRED technology thus circumvents major hurdle in mycobacteriophages manipulation: providing facile genetic approaches for addressing a multitude of questions in mycobacteriophages biology (Hatfull, 2010).

They have also provided insights into viral diversity and the evolutionary mechanisms that generate them and offer potential for the development of novel methodologies for the diagnosis, prevention, and treatment of these diseases as well as revealing interesting biological features of their unusual bacterial hosts (Hatfull, 2006; Piuri *et al.,* 2009).

Phage-based systems for the diagnosis of tuberculosis infections have also been described. There is currently one commercial manufacturer of phage-based tests on the market, Biotec Laboratories Limited (Ipswich, Suffolk, UK), which produces FASTPlaqueTB™ that detects *M. tuberculosis* in sputum samples and FASTPlaque-Response™ that detects rifampicin resistance in smear-positive sputum specimens containing *M. tuberculosis* but even after several modifications, the assays are not ready to be used (Trollip *et al.,* 2009).

Another method employs the use of recombinant mycobacteriophages, such as TM4 and D29, carrying the luciferase reporter gene (Jacobs *et al.,* 1993; Sarkis *et al.,* 1995). These FFlux reporter phages produce light when they infect *M. tuberculosis* and can be used to evaluate empirical drug susceptibility profiles by inclusion of antibiotics in the assay (Riska *et al.,* 1997; Riska *et al.,* 1998; Riska *et al.,* 1999; Banaiee *et al.,* 2001;). All these assays fell short in their sensitivity to be used for diagnosis. More recently, a new method for detecting drug resistant strains of *M. tuberculosis* was developed using a TM4 mycobacteriophage phAE87::hsp60-EGFP (EGFP-phage) engineered to contain the gene encoding a modified Green Fluorescent Protein. The method obtained a similar percentage of specificity and sensitivity in detecting isoniazid, rifampicin and streptomycin resistance in 155 strains of *M. tuberculosis*, when compared with the proportion method, as the reference standard. The authors suggest that the EGFP-phage method has the potential to be a valuable rapid and economic screen for detecting drug resistant tuberculosis if the procedure can be simplified (Rondón *et al.,* 2011).

Phage-based diagnostic tests are still at an early stage of their development; in future year's further technical innovation may improve their sensitivity, speed or convenience.

4.3. Mycobacteriophage Ms6

Ms6 is a temperate mycobacteriophage that infects *M.* smegmatis with ability to form stable lysogens. It was isolated by spontaneously induced *M. smegmatis HB5688* (Portugal *et al.,* 1989; Anes *et al.,* 1992). Ms6 is a double strand DNA phage and the length of the genome is over 50 Kb with a GC content of 62%. It is composed of an isometric polyhedral head with 80 nm in diameter, hexagonal shape, and a long non-contractile tail with 210 nm in length which allowed its classification in the *Siphoviridae* family (Portugal *et al.,* 1989) (Fig. 7).

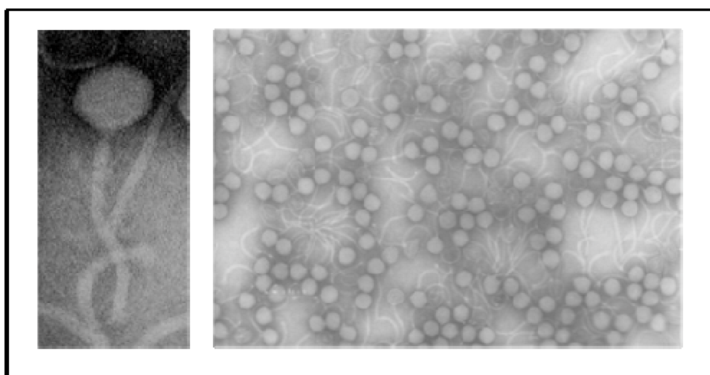

Figure 7. Mycobacteriophage Ms6 (electron microscopy picture).

Although the complete analysis of the nucleotide sequence is not yet available, some regions are already characterized. The site specific integration locus was identified within a 4.8 kb BglII Ms6 DNA fragment. The integrase gene encodes a protein of 372 aminoacids that drives integration into the 3' end of the *M. smegmatis* $tRNA^{Ala}$ gene. The core site, a fragment of 26 bp, where the recombination between the phage DNA and the bacterial genome occurs is positioned 98 bp upstream of the integrase gene (Freitas-Vieira *et al.,* 1998).

1673 bp downstream of the integrase gene and in the opposite direction, it was identified the gene *pin* coding for a protein involved in a superinfection exclusion mechanism. Work developed on the Ms6 Pin protein suggested that expression of this protein did not prevent Ms6 adsorption but blocks some early step following infection, at a stage between adsorption of the phage and DNA injection (Pimentel, 1999).

In 2002 Garcia *et al.,* described the genetic organization of the lysis module of mycobacteriophage Ms6 following the identification of a strong promoter region (P_{lys}). The authors reported that transcription of the lysis genes is driven by two σ^{70} like promoters that are recognized by the host RNA polymerase. A leader sequence of 214 bp was identified between this promoter region and the first lysis gene, in which was detected a transcription termination signal suggesting that an antitermination mechanism is involved in the regulation of Ms6 lysis genes transcription. The DNA sequence of the P_{lys} downstream region revealed the presence a lytic cassette composed of five genes, named *gp1* through *gp5* (Fig. 8).

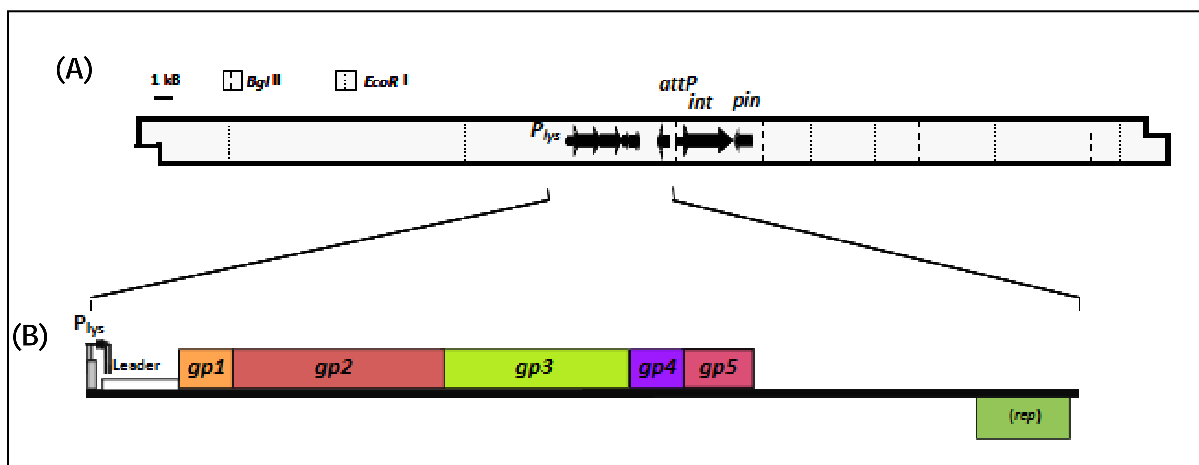

Figure 8. (A) Schematic representation of P_{lys} on the 57-kb Ms6 DNA. (B) Genetic organization of the Ms6 lysis locus. Figure adapted from Garcia *et al.,* 2002.

gp1, of 231 bp, encodes a protein of 77 amino acids that was very recently identified as a chaperone-like protein (Catalão *et al.,* 2010). Several physical and predicted structural characteristics of Gp1 are consistent with those of chaperones, in particular with the type III secretion system (TTS) chaperones that although do not exhibit amino acid sequence similarities, have in common physical characteristics, including a low molecular weight (< 15 kDa), an acidic pI (< 5), a predicted amphipathic helix near their C-terminal end and are encoded adjacently to their cognate effectors (Page & Parsot 2002; Feldman & Cornelis 2003).

In addition, Catalão *et al.,* (2010) showed that the Gp1 protein interacts with the N-terminal sequence (60 amino acids) of the Ms6 endolysin, its effector. Determination of the growth parameters of an Ms6 mutant phage deleted in *gp*1 gene showed that although Gp1 is not essential for plaque formation, it is necessary to achieve an efficient lysis, since its absence

results in a decrease of the burst size. Moreover, absence of Gp1 in a *M. smegmatis* infection results in a decrease of the full form of the endolysin.

A BLAST search using the GP1 amino acid sequence identified Gp1 homologous only in mycobacteriophages genomes and according to the recent analysis of mycobacteriophage genomes were grouped in Pham1480. Members of this gene phamily are present in the lysis cassette of mycobacteriophages that belong to subcluster A1, cluster E, subcluster F1 (Hatfull *et al.,* 2010; Henry *et al.,* 2010). Ms6 Gp1 showed the highest similarity to homologous proteins from mycobacteriophages belonging to subcluster F1. The localization of this gene family in the lytic cassette is highly diverse, in some cases the gene is overlapped with the predicted endolysin as it happens with Ms6 Gp1, in others the gene is separated from the endolysin gene by one intervening gene that code for putative homing endonuclease HNH motifs, while in some mycobacteriophages, this gene is apart from the lysin gene and distant in the genome (Hatfull *et al.,* 2010).

The ***gp2 (lysA)***, with 1152 bp, starts at a GTG codon that overlaps the *gp1* TGA codon, in a different reading frame. *gp2* or *lysA* was identified as encoding the endolysin protein (Garcia *et al.,* 2002). A search for conserved domains showed that Ms6 LysA holds a central peptidoglycan recognition protein (PGRP) conserved domain (cd06583), localized between amino acid residues 168 and 312. PGRPs are pattern recognition receptors that bind, and in certain cases, hydrolyze peptidoglycan of bacterial cell walls. This family includes Zn-dependent *N*-acetylmuramoyl-L-alanine amidases (EC: 3.5.1.28) which cleave the amide bond between *N*-acetylmuramoyl and L-amino acids, preferentially D-lactyl-L-Ala, in bacterial cell walls (Catalão *et al.,* 2011b). Piechota *et al.,* (unpublished work) demonstrated that LysA was able to cleave the bond between D-muramic acid of NGM (or NAG) and L-alanine by observing the release of up to 70% of the diaminopimelic acid present in purified mycobacterial cell wall, confirming the amidase activity of the enzyme.

Very recently, our group identified an embebbed gene designated lys_{241} (referring to the number of amino acids of the polypeptide) in the same reading frame of *lysA*. Even though deletion of the complete *lysA* nucleotide sequence revealed to be essential for *M. smegmatis* lysis, an Ms6 mutant lacking the longer ($lysin_{384}$) or the shorter ($lysin_{241}$) endolysin was shown to be viable, but defective in the normal timing, progression and completion of host cell lysis. In the absence of the longer form of LysA, lysis starts 30 min later than a wild type infection, and a reduction in the number of phage particles released occurs. When the smaller form (lysin241) is not produced, lysis starts 90 min latter than the wild type, although it does not have a significant effect in the number of phage particles released. These results indicate that both proteins are

necessary for complete and efficient lysis of *M. smegmatis* (Catalão *et al.,* 2011b). Additional studies demonstrated that both endolysins have peptidoglycan hydrolase activity and *E. coli* crude extracts containing $lysin_{384}$ or $lysin_{241}$ were shown to inhibit the growth of *Micrococcus luteus*, *Micrococcus pyogenes*, *Bacillus subtilis*, *Bacillus pumilus*, *Staphylococcus aureus*, *Mycobacterium smegmatis*, *Mycobacterium vaccae* and *Mycobacterium aurum* (Catalão *et al.,* 2011b).

LysA protein seems to be very well conserved among mycobacteriophages, since all mycobacteriophages genomes published so far encode a putative *lys*A-like gene and are part of the gene family Pham66-1. Although conserved, the LysA family appears to be highly diverse composed of subgenic modules with reasonably boundaries (Hatfull *et al.,* 2010) and may contain amidase, glycosidase or peptidase motifs (Payne *et al.,* 2009). The peculiar composition and structural features of the mycobacterial cell wall may reflect the extensive sequence divergence between these and other endolysins.

gp3 (***lysB***), with 996 bp, starts at an ATG codon that overlaps, in a different reading frame, the TGA stop codon of *lys*A and encodes a protein of 332 amino acids. Initially it was assigned for this putative protein a lysin function since it showed similarity to proteins with amidase and endochitinase activity and also due to its localization in the lysis cassette, next to the endolysin gene. A BLAST search has identified homologous proteins encoded in mycobacteriophages genomes as well as in phages that infects *Rhodococcus equi* (Summer *et al.,* 2011). These *lys*B-like genes have been grouped in the gene family Pham67 (Hatful *et al.,* 2010) and sequence analysis of this family showed that these proteins are highly diverse, but share the conserved pentapeptide motif GXSXG (Jaeger *et al.,* 1994; Arpigny & Jaeger, 1999; Bornscheuer, 2002).

gp4 (***hol***) begins with an ATG start codon located 10 nucleotides downstream of *gp3* stop codon and encodes a 77 amino acid polypeptide. The gene product of gp4 was identified as the Ms6 holin as it shares characteristics of class II holins, and can complement an S^{λ} defective mutant (Garcia *et al.,* 2002). A more recent characterization of Ms6 Gp4 function, suggests that Gp4 might function as a pinholin, since the first TMD has characteristics of a SAR domain with a high percentage of weakly hydrophobic or polar residues (Catalão *et al.,* 2011a) and expression in *E. coli* of LysA and Gp4 does not result in lysis (Garcia *et al.,* 2002). Noteworthy is the fact that deletion of gene *gp4* in the Ms6 genome results in a viable phage but with an anticipated time of lysis, contrary to what is described for proteins with holin function.

gp5, with 372 bp, starts at an ATG codon that overlaps the *hol* TGA stop codon, in a different reading frame. Analysis of the amino acid sequence predicted a TMD at the N-terminal region and a very highly charged and hydrophilic C-terminal domain, showing some holin features. In fact, deletion of *gp5* from the Ms6 genome resulted in a viable phage showing a delayed time of lysis, confirming that *gp5* has a role in the timing of lysis. However, contrary to Gp4, Gp5 does not complement an S^{λ} defective mutant. Cross-linking experiments showed that Gp4 interacts with Gp5. Taking the results all together it is suggested that the combined action of Gp4 and Gp5 play the role of a holin. Expression of both proteins is necessary to affect a host cell lysis at the correct and programmed timing. Moreover, interaction of Gp5 with Gp4 may contribute to very precise adjustment of the timing of hole formation and to keep the infected cell productive allowing the assembly of more virions (Catalão *et al.,* 2011a).

According to the sequence similarity to putative holin genes from mycobacteriophages of subcluster F1, the Ms6 holin was included in the Pham95 together with *gp37* from Che9d from subcluster F2 (Hatfull *et al.,* 2010). Putative holin genes were also identified in mycobacteriophages from subcluster A2, cluster E and from cluster I, all belonging to Pham1981 (Hatfull *et al.,* 2010). Whenever a holin gene has been assigned, including Ms6, it is closely linked to *lysA*. A BLAST search using Ms6 Gp5 deduced amino acid sequence identified a number of putative proteins with unknown functions restricted to mycobacteriophages with a high degree of sequence identity that have been recently grouped in a mycobacteriophages gene family Pham96. This gene family was identified in phages belonging to subcluster A2, subcluster F1, cluster I and in singleton phage Omega (Hatfull *et al.,* 2010).

Although Ms6 genome is not fully sequenced, its lytic cassette and gene organization is closely related to phages included in cluster F, subcluster F1, which includes phages Che8, Boomer, PCM, Fruitloop, Llij, Pacc40, Ramsey and Tweety (Hatfull *et al.,* 2010).

Of note is the fact that the lysis module of Ms6 and of the majority of mycobacteriophages, presents a gene (*lys*B) exclusive of phages infecting bacteria with similar cell wall (mycobacteria and rhodococcus). Its presence in the lysis module suggests that the gene product has a role in cell lysis. The observation of lipolytic domains in the aminoacidic sequence raises the possibility of an activity on the lipid contents of the mycobacteria cell envelope.

5. Lipolytic enzymes

5.1. General enzyme characterization

The term 'lipolytic enzymes' comprises lipases (EC 3.1.1.3), carboxylesterases (esterases, EC 3.1.1.1) (Arpigny & Jaeger 1999; Jaeger *et al.,* 1999; Bornscheuer, 2002) and cutinases (EC 3.1.1.74) (Carvalho *et al.,* 1999; Longhi & Cambillau 1999). Although the overall amino acidic sequence similarity of lipolytic enzymes is low, their molecular masses vary from 20 to 60 kDa, operating on substrates with completely different chemical or physicochemical properties. All lipolytic enzymes share a comparable 3D fold, showing the characteristic α/β-hydrolase fold, which is an eight-stranded mostly parallel α/β structure (Holmquist, 2000) and almost all belong to the class of serine hydrolases. The catalytic machinery of these proteins is composed by a nucleophile, an acid and a histidine where the active site serine is invariably located at the C-terminal end of one of the beta strands in an extremely sharp turn towards the next helix, the nucleophile elbow (Egmond, 2000). The catalytic triad is composed of Ser-Asp-His (Glu instead of His in some lipases) and usually a consensus sequence Gly-X-Ser-X-Gly is also found around the active site serine (Wei *et al.*, 1995).

Lipolytic enzymes have attracted an enormous attention due to their potential application in biotechnology and because some are involved in pathogenesis of many bacteria The lipolytic enzymes are one of the known virulence factors in many bacteria such as *Pseudomonas cepacia*, *Staphyloccocus aureus* (Lonon *et al.,* 1988; Rollof *et al.,* 1988), and in fungal species like *Alternaria brassicicola*, *Candida albicans* and *Fusarium graminearum* (Tsuboi *et al.,* 1996; Berto *et al.,* 1999).

5.2. Lipases *vs* carboxylesterases

The two major classes of lipolytic enzymes are the lipases and the carboxylesterases. It was demonstrated by Sarda (1958) and Desnuelle (1961) that the fundamental difference between carboxylesterase and lipase activity is based on the phenomenon of "interfacial activation", i.e., activity of lipases is enhanced on insoluble substrates (such as emulsions) (Rogalska *et al.,* 1997). Whereas carboxylesterases obey classical Michaelis-Menten kinetics, lipases need a minimum substrate concentration before high activity is observed. Structure elucidation revealed that this interfacial activation is due to a surface loop- the lid domain- that covers the active site, which is inaccessible to the solvent. This lid undergoes a conformational exchange

in a lipid-water interface, making the active site accessible, which explains the phenomenon of interfacial activation (Rogalska *et al.,* 1997). Furthermore, lipases prefer water-insoluble substrates, typically triglycerides composed of long-chain fatty acids, whereas carboxylesterases preferentially hydrolyze "simple" esters, usually triglycerides bearing fatty acids shorter than C_6. Both enzymes have been shown to be stable and active in organic solvents, but this feature is more pronounced with lipases (Jaeger, 1994; Arpigny & Jaeger, 1999) performed a classification of lipases and carboxylesterases into 8 families based on their amino acid sequence similarity and their conserved motifs. Despite a highly conserved tertiary fold and sequence similarities, lipolytic enzymes display a wide diversity of properties and of relatedness to other protein families (Arpigny & Jaeger 1999).

5.3. Cutinases

Cutinases are extracellular enzymes that have been found to occur in phytopathogenic bacteria and fungi (Schué *et al.,* 2010) and catalyze the cleavage of ester bonds of cutin, resulting in the release of cutin monomers, polyester that protects plant leaves (Carvalho *et al.,* 1999; Kolatthkudy, 2001). This family is one of the smallest members of the serine hydrolase family (Egmond & Vlieg 2000). In addition to the GXSXG motif, present in lipolytic enzymes, cutinases present the common motifs P-x-[STA]-x-[LIV]-[IVT]-x-[GS]-G-Y-**S**-[QL]-G and C-x -(3)-**D**-x-[iv]-c-x-G-[gst]-x(2)-[LIVM]-x(2,3)-**H** (www.expasy.ch/cgibin/nicedoc.pl?PDOC00 140) (Parker *et al.,* 2007). The most well known and most fully characterized cutinase is the cutinase from *Fusarim solani* (Longhi *et al.,* 1999). This enzyme has been found to degrade a wide range of substrates, including cutin, carboxylic esters, triacylglycerols (TAGs) (Mannese *et al.,* 1995), phospholipids (Parker *et al.,* 2007) with both long and short-chain fatty acids and is also active in soluble substrates, such as short-chain *p*-nitrophenyl (*p*NP) esters. This characteristic is probably due to the fact that unlike several lipases, which require an interface, the catalytic site of cutinase is not covered by a lid and therefore remains accessible to the substrate in solution (Martinez *et al.,* 1992). The biochemical properties of cutinases are, therefore, challenging the classical definition of lipases and esterases, being considered as intermediate between lipases and esterases (Carvalho *et al.,* 1999; Longhi & Cambillau 1999; Egmond & Vlieg 2000).

5.4. Lipolytic enzymes in mycobacteria

Genes encoding lipolytic enzymes have also been annotated in mycobacteria genomes. *Mycobacterium tuberculosis* is a pathogen that can persist for decades in an infected host in a dormant state, without causing disease until the host's immune system is weakened**.** Before

entering in this state, mycobacteria accumulate lipids, in inclusion bodies, from the host cell membrane degradation and form de novo synthesis. During the reactivation phase, these stored lipids are hydrolyzed and an active infection is established. The availability of the complete genome sequence of *M. tuberculosis* strain H37Rv (Cole *et al.,* 1998), allowed the identification of 250 genes encoding proteins involved in lipid metabolism. From these, 24 genes code for lipolytic enzymes which were classified into a family called the "Lip Family" (LipC to LipZ). This classification was based on the presence of the consensus sequence GXSXG, not allowing the distinction between lipases and esterases. Only for LipY was experimentally determined its lipolytic activity (Deb *et al.*, 2006). Genes encoding homologous proteins to cutinase of *F. solani* have also been reported to occur in various environmental mycobacterial species, such as *Mycobacterium vanbaalenii* and *Mycobacterium* sp. strain KMS, which have frequently been isolated from soils where dead plant organic matter accumulates (Belbahri *et al.,* 2008). More surprisingly, similar genes have been detected in mycobacterial species that do not naturally encounter cutin polymers, such as human pathogens *M. tuberculosis* and *M. bovis* (Parker *et al.,* 2007).

From the *M. tuberculosis* genome annotation, 7 cutinase-like proteins were identified, presenting the above cutinase motif. The presence of such enzymes suggests that they play an important role and that the use of host lipids for growth *in vivo* may be one of the major characteristics of the *M. tuberculosis* life cycle (Parker *et al.,* 2007; Côtes *et al.,* 2008; West *el al.*, 2009). In pathogens that do not encounter cutin, the presence of these enzymes likely represents an evolutionary divergence, and activity on other substrates, such as the phospholipids, is likely to be more important (Parker *et al.,* 2007; Côtes *et al.,* 2008; West *el al.*, 2009). Lipases may not be the direct targets involved in pathogenesis but may contribute for the survival of the mycobacteria. It can be considered that new drugs targeting these enzymes could be used to alter the metabolism of the mycobacterial in order to allow a better access of the drugs actually available to treat the disease (Côtes *et al.,* 2008).

6. Phage therapy

Phages were first described by Felix d'Herelle in 1917, although their antibacterial activity had been independently recognized by Hankin in 1896, Gamaleya in 1898 and Twort in 1915. D'Herelle was quick to realize the potential for therapeutic applications of phages, and he

championed the concept of using them to treat bacterial infections (Sulakvelidze *et al.*, 2001). The potential use of phages to treat infections attracted the attention of large pharmaceutical companies in the United States in the 1929s and 1930s (Merril *et al.*, 2003). For example, E. R. Squibb & Sons sold a phage filtrate preparation for *Staphylococcus* sp. Phagoid Laboratories, Inc., marketed an array of phage-based products, including Phagoid-Staphylococcus, Phagoid-Gomococcus, and Phagoid-Arthritis (McKinstry & Edgar, 2005). It was not until 1940 that the nature of phages was clearly confirmed by electron microscopy and began the "modern" study of phages; however the discovery of antibiotics has diverted research attention away from phage therapy in the USA and elsewhere (McKinstry & Edgar, 2005), except in the former Soviet Union and Eastern Europe. In these countries, particularly in centres such as the Eliava Institute of Bacteriophages, Microbiology and Virology in Tbillisi, Georgia and the Institute of Immunology and Experimental Therapy in Wroclaw, Poland (Sulakvelidze & Kutner, 2005; Gorski *et al.,* 2007), bacteriophages have continued to been used clinically for the treatment of bacterial infections continuously since 1919. The Eliava Institute has developed its bacteriophage collection over many decades, and producing a range of phage preparations in a variety of pharmaceutical forms that are able to be administered topically, orally, rectally, by inhalation or by injection.

The current increasing incidence of antibiotic resistance in bacterial pathogens has justified a reassessment of the value of phages as antibacterial agents for medical and veterinary applications (Projan & Shales 2004; Norrby *et al.,* 2005). This problem has intensified the need for the implementation of new effective measure to control infections by bacterial pathogens. Since the early 1990s the number of animal studies has expanded rapidly and prophylactic and/or therapeutic benefits have been reported for a range of experimental infections including vancomycin-resistant *Enterococcus faecium* (Biswas *et al.,* 2002), *P. aeruginosa* (Watanabe *et al.,* 2007), *Mycobacterium avium* (Danelishvilli *et al.,* 2006), *E. coli* (Nishikawa *et al.,* 2008) including Extended spectrum Beta-lactamase strains (Wang *et al.,* 2006) among others. A high number of recent publication show promising success for phage therapy and human clinical trials with phages have been initiated by various phage companies to treat ear infections, leg ulcers and burn wounds (Fortuna *et al.,* 2008).

The application of mycobacteriophage to treat diseases has comprehensively received less attention. Early attempts to treat laboratory animals infected with *M. bovis* BCG and *M. tuberculosis* were not successful (Hauduroy & Rosset 1963; Mankiewicz & Beland 1964). Killing intracellular pathogens such as *M. tuberculosis* presents a tough challenge because in order to infect the target bacilli the phage need to transverse the mammalian cell membrane and

survive in adverse intra-cellular environments. Since phages, in contrast to antimicrobials, are not diffusible across membranes, strategies need to be devised to deliver the phage to the intracellular pathogen. A second challenge to phage therapy is the presence of granulomas which might prove impenetrable to the bacteriophage (Bowman *et al.,* 1972) and thus novel phage delivery systems are required. A possible strategy has recently been identified where mycobacteriophage TM4 can be delivered by a transiently-infected nonvirulent mycobacterium to kill both *M. tuberculosis* and *M. avium* inside the macrophage (Ford *et al.,* 1998; Foley-Thomas *et al.,* 1995). Macrophage cell lines infected with *M. tuberculosis* or *M. avium* were treated by the addition of *M. smegmatis* infected with mycobacteriophage TM4. The results showed that when delivered to the site where the pathogenic bacterium resides inside the macrophages, TM4 was effective in killing *M. avium* and, even more significantly, *M. tuberculosis* (Broxmeyer *et al.,* 2002). Another study demonstrated that infected-mycobacteria macrophages could phagocyte mycobacteriophage D29 resulting in an effective lysis of intracellular mycobacteria without any deliver system (Peng *et al.,* 2006). Phage therapy might be more readily applicable to less visceral mycobacterial diseases where the site of infection is accessible, such as in Buruli ulcer caused by *M. marinum*. However, no such studies have so far been reported. Further research is needed to overcome the existing technical barriers and enable the development of effective therapeutic tools (McNerney & Traoré 2005).

Another example of the use of bacteriophages is the research into the utilization of phage lysins as therapeutic agents. As these enzymes break down the cell wall, they have the potential to be used as therapeutic agents (O'Flaherty *et al.*, 2009). They have advantages over antibiotics in that they possess the host specificity of phages and so do not adversely affect normal microflora; there is less opportunity for resistance to emerge and they kill colonizing pathogens on mucosal surfaces (Fischetti, 2005). Because lysins target peptidoglycan-associated carbohydrates as binding epitopes, the evolution of resistance to lysins may be precluded, as these moieties are often essential for viability (Fischetti *et al.,* 2006). Their major disadvantage is that they are unable to penetrate the outer membrane of Gram-negative cells and so their therapeutic activity is almost entirely directed at Gram-positive infections. Although the capacity of a phage endolysin to kill bacteria was first reported in 1957, it was not until 2001 that Fischetti and co-workers demonstrated that a purified recombinant endolysin can be used as therapeutic agent to prevent or reduce the colonization of mucosal surfaces with group A streptococci in mice (Nelson *et al.,* 2001). This strategy has been extended and proved potentially useful to control sepsis or other infections produced by several Gram-positive

bacteria, like *Enterococcus feacali* and *E. faecium* (Yoong *et al.,* 2004), *Clostridium perfringens* (Zimmer *et al.,* 2002) or group B streptococci (Cheng *et al.,* 2005). This collection of successful reports employing phage-encoded bacterial cell wall lytic enzymes to eliminate pathogenic bacteria has led to the designation of these muralytic enzymes as enzybiotics (Nelson *et al.,* 2001).

Mycobacteria are likely to be intractable to exogenously added endolysins, as it happens to Gram-negative bacteria, because of their mycolic-acid-rich outer membrane (Payne *et al.,* 2009) and the complexity of the cell wall; enzymes that attack the mycobacterial envelope from the outside are needed as a novel therapeutic method to kill mycobacteria.

It is increasingly clear that the future of lysin development will also focus on biotechnology and food biocontrol applications. One example is secretion of recombinant lysins from *Lactococcus lactis* starter cultures, used in the fermentation of milk, to combat *L. monocytogenes*, which can contaminate dairy products (Gaeng *et al.,* 2000). Another example is the creation of transgenic cattle that secrete a recombinant lysin-like hydrolase, lysostaphin, into their milk to protect against *Staphylococcus aureus* mastitis (Kerr *et al.,* 2001).

Although the majority of reports are focused on the lysins, there are other phage components that may, directly or indirectly, lead to valuable new therapeutic agents. For example, the lysis system of certain small genome viruses, such as Qβ and φX174, encode alternative non-lysin lytic agents that block the enzymes responsible for early steps in bacterial peptidoglycan biosynthesis (Bernhardt *et al.,* 2001b; Bugg *et al.,* 2006).

Despite the advantages and optimism regarding the realization of phage therapy, both past and present, many obstacles remain. The effective use of phages to treat infectious disease will require a more thorough understanding of phage-bacterium-mammal interaction *in vivo*. Pharmacokinetic models may help to predict the optimal timing of inoculation for phage therapy application. Growth and purification standards need to be established for any phage to be selected for therapeutical use. The presence of toxin genes in some phages requires the DNA sequencing of any therapeutic phages as part of a rigorous characterization scheme. For phage therapy to be effective in a clinical setting, a swift assessment of bacterial susceptibility will be necessary. However exploring the mechanisms by which mycobacteriophages lysis their hosts will certainly contribute to identify new therapeutic targets and thus to the development of efficient antimycobacterial agents

A better understanding of phage biology and phage-bacterium-host interactions and the ability to genetically manipulate these viruses may allow for the realization of the early promise of phage therapy (McKintry & Edgar 2005).

OBJECTIVES

The main goal of this thesis is to study the product of *lys*B gene of mycobacteriophage Ms6 lytic cassete. The presence of a conserved motif characteristic of lipolytic enzymes present in the aminoacidic sequence of LysB and it homologous proteins from other mycobacteriophages, was the basis of the research presented in this thesis.

In order to achieve this goal it is necessary:

- Identify and characterize *lys*B gene;
- Confirm the lipolytic activity assigned to LysB due to the presence of the conserved motif and characterize LysB activity (substrate affinity, inhibiting substrates, active site);
- Localize LysB target in the mycobacteria cell envelope;
- Determine the importance of Lys during the infection cycle of mycobacteriophage Ms6.

Due to the increasing prevalence of multi-resistant bacteria to the current antibiotics, it is necessary to search for different means to fight this growing problem spread throughout the world. Phages and phage products have been recently reevaluated as potential antibacterial agents for medical and veterinary applications. Our overarching goal is not only to provide information regarding the mechanisms involved in mycobacteriophage lysis but also to present new possible targets to fight one of the most important human pathogens, *Mycobacterium tuberculosis*.

2

The lytic cassette of mycobacteriophage Ms6 encodes an enzyme with lipolytic activity

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Summary

dsDNA bacteriophages use the dual system endolysin–holin to achieve lysis of their bacterial host. In addition to these two essential genes, some bacteriophages encode additional proteins within their lysis module. In this report, we describe the activity of a protein encoded by gene *lysB* from the mycobacteriophage Ms6. lysB is localized within the lysis cassette, between the endolysin gene (lysA) and the holin gene (*hol*). Analysis of the deduced amino acid sequence of LysB revealed the presence of a conserved motif (Gly-Tyr-Ser-Gln-Gly) characteristic of enzymes with lipolytic activity. A BLAST search within the sequences of protein databases revealed significant similarities to other putative proteins that are encoded by mycobacteriophages only, indicating that LysB and those proteins may be specific to their mycobacterial hosts. A screening for His_6-LysB activity on esterase and lipase substrates confirmed the lipolytic activity. Examination of the kinetic parameters of recombinant His6-LysB for the hydrolysis of *p*-nitrophenyl esters indicated that although this protein could use a wide range of chain length substrates (C_4–C_{18}), it presents a higher affinity for *p*-nitrophenyl esters of longer chain length (C_{16} and C_{18}). Using *p*-nitrophenyl butyrate as a substrate, the enzyme showed optimal activity at 23 °C and pH 7.5–8.0. Activity was increased in the presence of Ca^{2+} and Mn^{2+}. To the best of our knowledge, this is the first description of a protein with lipolytic activity encoded within a bacteriophage.

Introduction

Bacteriophages are viruses that infect bacterial cells. For their own survival they need to infect sensitive bacteria, where they replicate and produce new viral particles. At the end of the lytic cycle, phages need to exit the bacteria, and liberate the progeny phage into the environment, and thus infect new bacteria. To achieve this, dsDNA phages have evolved a lytic system that compromises the integrity of the cell wall, resulting in bacterial lysis (Young, 1992; Young *et al.*, 2000). This system consists of at least two genes: an endolysin that targets the cell wall, and is designed to attack one of the four major bonds in the peptidoglycan; and a holin, which controls the timing of lysis, and at a genetically defined time allows the endolysin to reach its target, leading to disruption of the cell wall, and release of the new progeny virions (Young, 1992; Young *et al.*, 2000; Grundling *et al.*, 2001). In addition to these two essential genes, the lytic cassette of some bacteriophages may contain additional genes that may, or may not, be essential for lysis. The l cassette includes the R*z* and *Rz1* lysis genes, which encode an integral inner-membrane protein and an outer-membrane lipoprotein; under usual conditions these proteins are not essential for lysis (Young, 2005; Summer *et al.*, 2007). Rz/Rz1 equivalents have been recently identified in phages of Gram-negative hosts (Summer *et al.*, 2007); however, a clear function has not yet been established. In addition, dsDNA phages often encode a holin inhibitor or antiholin, which is a negative regulator of holins (Young, 2002). The lysis module of the mycobacteriophage Ms6, a temperate phage that infects Mycobacterium smegmatis (Portugal *et al.*, 1989), has previously been identified, and consists of five genes (Garcia *et al.*, 2002). In addition to the endolysin (*lys*A) and the holin (*hol*) genes, the Ms6 lytic cassette comprises three additional genes whose functions have not yet been identified. Between *lys*A and *hol*, the gene *lysB* (gp3) encodes a protein of 332 aa. Analysis of the LysB deduced amino acid sequence has revealed the presence of a conserved pentapeptide Gly-Tyr-Ser-Gln-Gly motif, which matches the Gly-X-Ser-X-Gly consensus motif that is characteristic of lipolytic enzymes (Jaeger *et al.*, 1994; Arpigny & Jaeger, 1999; Bornscheuer, 2002). The term 'lipolytic enzymes' comprises lipases (EC 3.1.1.3), carboxylesterases (EC 3.1.1.1) (Arpigny & Jaeger, 1999; Jaeger *et al.*, 1999; Bornscheuer, 2002) and cutinases (EC 3.1.1.74) (Carvalho *et al.*, 1999; Longhi & Cambillau, 1999). Lipases are, by definition, enzymes that have the ability to hydrolyse long-chain acylglycerols (≥C10), whereas esterases hydrolyse ester substrates with short-chain fatty acids (≤C10) (Arpigny & Jaeger, 1999; Jaeger *et al.*, 1999; Bornscheuer, 2002). Cutinases hydrolyse the water-insoluble biopolyester cutin, a component of the waxy exterior layer of plants. In addition to cutin, cutinase substrates include a wide variety of esters ranging from soluble *p*-nitrophenyl (pNP) esters to insoluble long-chain triglycerides (Carvalho *et al.*, 1999; Longhi & Cambillau, 1999). Although the overall sequence similarity of lipolytic enzymes is low, and their molecular masses vary from 20 to 60 kDa, all these enzymes share a

comparable 3D fold, which is known as the α/β hydrolase fold (Holmquist, 2000). Activity of these enzymes relies mainly on a catalytic triad formed by a nucleophilic Ser residue, usually appearing in the conserved pentapeptide Gly-X-Ser-X-Gly consensus motif, an acidic residue (Asp or Glu), and a His; in this order, these form the catalytic triad (Arpigny & Jaeger, 1999; Carvalho *et al.*, 1999; Bornscheuer, 2002; Gupta *et al.*, 2004). In this study, we characterize the gene product of *lysB*, and demonstrate that LysB is an enzyme with lipolytic activity. We also present data that show the presence of genes encoding similar proteins within the genome of mycobacteriophages. To the best of our knowledge, this is the first description of a lipolytic enzyme encoded within a bacteriophage genome, and it indicates that the lytic cassette of Ms6 mycobacteriophage is different from the majority of the lytic cassettes described to date. The recombinant lipolytic enzyme was characterized with regard to optimum reaction conditions, substrate specificity, and effect of inhibitors and metal ions.

Methods

Bacterial strains and media

Plasmid-transformed *Escherichia coli* JM109 was grown at 37 °C in Luria–Bertani (LB) broth or agar containing 100 μg ampicillin ml^{-1}.

DNA manipulation, cloning and sequence analysis.

DNA amplification by PCR, plasmid isolation, electrophoresis and *E. coli* DNA transformation were carried out using standard techniques (Sambrook & Russell, 2001). Restriction enzymes and T4 DNA ligase (New England Biolabs) were used according to the supplier's recommendations. In order to construct plasmid pMP302, the gene encoding *lysB* was PCR amplified with primers (restriction sites underlined) PrLys3A (59-GGGCCACTG GATCCCGCATCGACG-39) and PrLys3C (59-GCATGGGTCGACCTCCTATG TGCG-39), using Ms6 DNA as a template. The PCR product was purified with the MinElute PCR Purification kit (Qiagen), digested with *Bam*HI and *Sal*I, and cloned into the same restriction sites of the expression vector pQE30 (Qiagen). The resulting plasmid was transformed into *E. coli* JM109 cells. The BLAST 2.0 program at NCBI was used for similarity searches of protein sequences (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence alignments were performed using CLUSTALW multiple sequence alignment software (http://www.ebi.ac.uk/clustalw/). The physicochemical parameters of the deduced amino acid sequence were determined by using the ProtParam tool at ExPASy (http://www.expasy.org).

Expression and purification of LysB protein.

E. coli JM109 (pMP302) was grown in LB medium to an OD_{600} of 0.6, and expression of the recombinant His_6-LysB was induced for 4 h with the addition of IPTG to a final concentration of 1 mM. Bacterial cells were harvested by centrifugation, washed, resuspended in 50 mM Tris/HCl (pH 7.5) supplemented with a cocktail of protease inhibitors (Calbiochem), and disrupted by passage through a French pressure cell press. Cell debris was removed by centrifugation, and the recombinant His_6-LysB present in the supernatant was purified by passage through a Ni-NTA column (Qiagen), according to the manufacturer's instructions. Purified protein was analysed by SDS-PAGE, followed by Coomassie blue staining and Western blot. The protein was detected with horseradish-peroxidase-conjugated anti-His monoclonal antibody (Roche). Protein concentration was determined by the Bradford method (Bradford, 1976), using BSA as a standard.

Enzymic activity assays.

The esterase and lipase activities of the purified His_6-LysB were examined on LB agar plates containing substrates as follows: (**a**) 1% (v/v) tributyrin (Sigma), (**b**) 1% (v/v) triolein (Sigma) and 0.001% (w/v) rhodamine B, (**c**) 1% (v/v) Tween 80 with 1 mM $CaCl_2$, or (**d**) 1% (v/v) Tween 20 with 1 mM $CaCl_2$ (Kouker & Jaeger, 1987; Nikoleit *et al.*, 1995). A 100 µg quantity of His_6-LysB was spotted on the different LB media, and plates were incubated at 37 °C for 24 and/or 48 h. Enzymic activity was indicated by the formation of a clear zone on tributyrin plates, by a fluorescent halo visible on irradiation with a Ultraviolet lamp on triolein plates, and by the formation of a white precipitate on Tween plates. For the specific detection of lipase activity, 100 µg His_6-LysB was incubated at room temperature with 100 µg 1,2-O-dilauryl-*rac*-glycero-3-glutaric acid resorufin ester (Sigma) in 1 ml of 100 mM Tris/HCl pH 7.5 and 0.2% Triton X-100. The release of resorufin was monitored by measuring the change in the absorbance at 572 nm (ΔA_{572} min^{-1}) over a period of 10 min (Schmidt *et al.*, 2004). The hydrolase activity of recombinant His_6-LysB was further examined on LB agar plates containing skim milk (1 %, w/v) or sheep blood erythrocytes (7 %, v/v). Plates were incubated at 37 °C with a spot of His_6-LysB (100 µg).

The substrate specificity of the purified His_6-LysB was measured using pNP esters (Sigma) with carbon chain length ranging from C_4 to C_{18}. The released pNP from the substrates *p*-nitrophenyl butyrate (pNPB; C_4), *p*-nitrophenyl caprylate (pNPC; C_8), *p*-nitrophenyl laurate (pNPL; C_{12}), *p*-nitrophenyl myristate (pNPM; C_{14}), *p*-nitrophenyl palmitate (pNPP; C_{16}) and *p*-nitrophenyl stearate (pNPS; C_{18}) was monitored using a spectrophotometric assay. The enzymic reaction was performed at room temperature (23 °C) in a final volume of 200 µl containing 60 µg

purified His_6-LysB ml^{-1}, 1 mM test substrate, and 0.2% Triton X-100 in 100 mM Tris/HCl buffer, pH 7.5 (standard assay conditions). The released pNP was monitored at 405 nm in a Microplate Reader model 680 (Bio-Rad), over a period of 30 min, and quantified using a calibration curve of pNP (0.5–250 μM). To determine the kinetic parameters, the enzymic assays were performed as described above using substrate concentrations ranging from 10 μM to 5 μM.

The effects of temperature and pH on the lipase activity were determined spectrophotometrically using pNPB (1 mM) as the substrate. The optimum temperature for lipase activity was determined by measuring the rate of reaction at temperatures ranging from 4 to 63 °C, at pH 7.5. The optimum pH for His_6-LysB activity was determined at room temperature (23 °C) using buffer containing 100 mM Tris/HCl and 0.2% Triton X-100, and adjusted to pH 5, 6.8, 7.5, 8, 8.8 and 9.5.

To test the effect of metal ions on the catalytic activity of His_6-LysB, the enzymic assay was performed at standard conditions using pNPB as the substrate, in the presence of one the following metal ions (5 mM): Ca^{2+}, Mg^{2+}, K^{+}, Cd^{2+}, Mn^{2+}, Hg^{2+} and Zn^{2+}. The effect of protease inhibitors was examined using EDTA (10 mM), PMSF (10 mM) and diethylpyrocarbonate (DEPC) (20 mM). The purified lipase was pre-incubated with the ions or inhibitors for 2 h at room temperature, and the residual lipase activity was measured. A negative control was performed in all experiments, using a reaction mixture of identical composition, except that LysB was omitted. All the assays were performed in triplicate. The kinetic parameters were calculated using the Sigma Plot SPSS Enzyme Kinetics module 1.1 software. One unit of enzyme activity was defined as the amount of enzyme that produced 1 μmol pNP min^{-1}.

Site-direct mutagenesis

Site-direct mutagenesis was performed with the QuickChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), according to manufacture's instructions. The plasmid pMP302 was used as a template for mutagenesis on the catalytic triad to change Ser168, Asp215, His246 or Asp306 to alanine. The oligonucleotides used are described in Table I. Introduction of the desired mutations was confirmed by plasmid sequencing. The mutant proteins were purified as described above for $LysB_{Wt}$. Characterization of mutated proteins activity was performed as described above with pNPB substrate

Table 1 – Oligunucleotides used to generate point mutations in *lysB*

Name	Sequence (5' to 3')
PrSer168Ala Fw	CACTGGCGGGATATGCGCAAGGCGCG
PrSer168Ala Rv	CGGGCCTTGCGCATATCCCGCCAGTG
PrAsp215Ala Fw	CACGTTTGGGCTGCTCACGGCGGCTCC
PrAsp215Ala Rv	GGAGCCGCCGTGAGCAGCCCAAACGTG
PrHis246Ala Fw	CGCGACTACGCCGCCCAAGGCGACCTG
PrHis246Ala Rv	CAGGTCGCCTTGGGCGGCGTAGTCGCG
PrAsp306Ala Fw	CCGGGCGATCCTCGCCGCCGGCATGTTC
PrAsp306Ala Rv	GAACATGCCGGCGGCGAGGATCGCCCGG

Results

Cloning and expression of the *lysB* gene

The DNA sequence encoding LysB was cloned into the expression vector pQE30 fused to an N-terminal His_6-tag, and the recombinant protein was expressed after induction with IPTG. Growth and viability of the recombinant strain *E. coli* JM109 (pMP302) was not affected, even after the addition of 2% $CHCl_3$ (data not shown). It has been shown for other phages that expression of a bacteriophage lysin gene in *E. coli* causes cellular lysis after permeabilization of the plasma membrane with chloroform (Chandry *et al.*, 1997; Henrich *et al.*, 1995); this effect is also observed with the lysin LysA of mycobacteriophage Ms6 (Garcia *et al.*, 2002). The result indicates that LysB is not an additional endolysin. SDS-PAGE analysis revealed the presence of a soluble His_6-tagged LysB with a molecular mass consistent with a predicted molecular mass of 38.3 kDa. This was confirmed by Western blot analysis using anti-His antibody, which recognizes the N-terminal His_6-tag on recombinant His_6-LysB (Fig. 1).

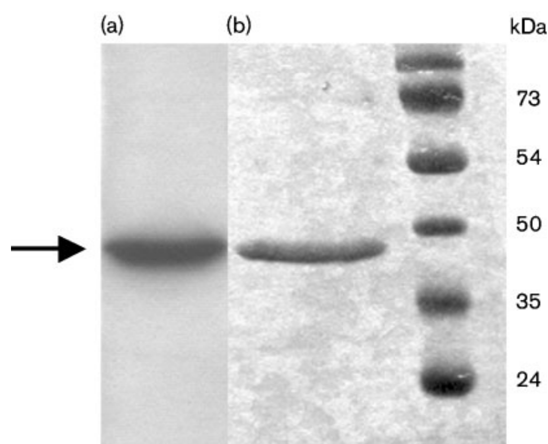

Figure 1. (A) Western-blot of purified His6-LysB; (B) Coomassie blue-stained SDS-PAGE of purified His_6-LysB. The sizes of the molecular mass markers are indicated on the right, and the position of the His_6-LysB protein is indicated by an arrow.

Analysis of the amino acid sequence of LysB

A BLAST search using the Ms6 LysB deduced amino acid sequence identified a number of mycobacteriophage putative proteins with a high degree of sequence identity. These putative proteins are localized within the lysis cassette of these mycobacteriophage genomes, and, due to their related amino acid sequences, they have been recently grouped in a mycobacteriophage gene family Pham9 (Hatfull *et al.*, 2006). The highest identity (88 %) was observed with the predicted amino acid sequence of Gp33 from mycobacteriophage Che8, a phage that also infects *M. smegmatis*.

A multiple alignment of sequences with greater than 40% identity (Fig. 2) revealed the presence of a conserved pentapeptide Gly-Tyr-Ser-Gln-Gly motif at positions 166–170 of the LysB amino acid sequence; this matches the characteristic Gly-X-Ser-X-Gly motif found in lipolytic enzymes. An important feature of the α/β hydrolase superfamily is the presence of Asp and His residues, which, together with the Ser residue, form the catalytic triad (Arpigny & Jaeger, 1999; Holmquist, 2000; Gupta *et al.*, 2004). LysB did not show significant amino acid sequence similarity with members of lipase families (data not shown); however, the alignments presented in Fig. 2 shows Asp and His residues conserved among the homologous proteins of the mycobacteriophages. Although three Asp residues (Asp215, Asp249 and Asp306) are totally conserved, Asp215 together with His246 might be good candidates for the catalytic triad, obeying the order Ser-Asp-His. Based on a comparison of the amino acid sequences, Arpigny & Jaeger (1999) classified bacterial lipolytic enzymes into eight families; however, LysB and the putative mycobacteriophage proteins do not belong to any of these families. Cutinases are not included in these eight bacterial lipolytic families, probably because these lipolytic enzymes were primarily described in fungus. Although genes coding for cutinases have been annotated in the genome of mycobacteria (Parker *et al.*, 2007), LysB and the homologous mycobacteriophage proteins do not fit the consensus pattern of cutinases around the Ser active residue. From the alignment represented in Fig. 2, we can observe conserved blocks among these mycobacteriophage protein sequences that are not present in any of the lipases families described to date; therefore, we suggest that they might form a new family of lipolytic enzymes.

No signal peptide (SignalP 3.0 server) was found, suggesting that LysB is not a secreted enzyme, as is the case with many lipases (Gupta *et al.*, 2004).

```
LysB  Ms6       93 PVLFTVCGTGVPWWVGP--DADTARAVEDQYLWQPIG--YPAAPFPMGRSI  139
Gp33  Che8      94 PVLITVCGTGVPWWIGP--DADTARAVEDKYLWQPIG--YPAAPFPMGKSI  140
Gp70  Corndog  101 PLFITVCGTGVPWWVGP--DADTARACEDKLFWQPIG--YPAKNFPMGKSI  147
Gp238 Bxz1      98 PVLFTVCGTGVPWWVGP--DAETARQVEDLYRWQPVG--YPAAPFPMGPSI  144
Gp9   Bxb1      81 PVLLTVSGTGVPWWVGP--DADVARRLGDVYLWRPVGPPYTAQAFPMGPSV  129
Gp52  Wildcat   86 PTLYTVHGTGVSMWDGP--PADCARRLLDKYRWQPVG-NYPASAFPMWPSI  133
Gp12  L5         4 PWLFTVHGTGQPDPLGPGLPADTARDVLDIYRWQPIG-NYPAAAFPMWPSV   53
Gp12  Bxz2      83 RWLFTVHGTGQADPLGPGLPADTARAVLDKYTWQPIG-NYPARAFPMWSSI  132
                     : ** *** .   **   *: **   *   *:*:*  *.*  ***  *:

LysB  Ms6      154 RERIETHGTALAGYSQGAVVLSEL 177  201 TWGNPNRELGHVWADHGGSPMAPSNTQGVS 230
Gp33  Che8     155 RERIETHGAALAGYSQGAVVVSEL 178  202 TWGNPNRELGHVWADHGGSPMAPSNTQGVS 231
Gp70  Corndog  168 RQRIERNGVVLGGYSQGAVVVSEL 191  215 TWGNPNREVGAVWPDYGGSPMASLTSQGVS 244
Gp238 Bxz1     165 RKQVERNGMVLAGYSQGAIVTSEV 188  212 AWGNPNREQGKAYPDAG-APLAAADSAGIT 240
Gp9   Bxb1     144 RRRIERYGLSMIGYSQGAIVTSEL 167  191 TFGNPMRETGKVWPDPGGQ-MPSAKSHGIA 219
Gp52  Wildcat  157 ----TPGKFTLAGYSQGAMVTSIV 176  200 TWGNPMREMGKAWTDGVG-AVAGQNNGGIA 218
Gp12  L5        68 LDADPYADFALAGYSQGAIVVGQV  91  115 FWGNPMRQKGFAHTDEWIHQVAASDTMGIL 144
Gp12  Bxz2     157 S-----GEVNLAGYSQGAVVVGQV 165  189 LWGNPMRQRGIAHFDEWIHPVAGPDSYGIL 218
                            :  ******:* . :            :*** *: * .  *     :.   . *:

LysB  Ms6      241 WRDYAHQGDLYACTEPGDTQEVRNAIWQIVRDLDLFT-GPDSLLAQVIELAQAPLPETIAITRA 303
Gp33  Che8     242 WRDYAHQGDLYACTEPGDTQEVRNAIWQIVRDLDLFT-GPDSLLAQVVELVQAPLPEAIAITKA 304
Gp70  Corndog  255 WRNYAHAGDLYAAAEPGDSQQDKNAIWQIIRDLNFFT-GTDSLLAQAIELSQMPIPRTIAAFKA 317
Gp238 Bxz1     251 WRNYAHQGDLYTATRPGESREDKVAIWQIVRGTNILS-GPDSLLRQFLEIAEAPVPNAIAAFQA 313
Gp9   Bxb1     230 WRNYAHKGDLYTDCE-GDSGEMKTAIYKVVMMSRVFS-GPDSILRQLLEIGVNPTFELIALIRA 291
Gp52  Wildcat  229 WRDYAHKGDLYTDCEFDDEGEYKRSVCKIVMGHNVFG-GPDSILRQVIELGLDPFGEAIPMIKA 291
Gp12  L5       158 VRDYAHDGDMYASIKEDDMHEYEVAIGRIVMSARRFIGGKDSVIAQLIELGQRPIWEGIAMARA 221
Gp12  Bxz2     232 IRDYAHAGDMYASITDGDKDEYKIAICKIVMTATDFYRGPNSVVSQLIELGQRPLTEGIAMALA 295
                    *:*** **:*:    .:  : . :: :::     :  * :*:: * :*:   *  . *.   *

LysB  Ms6     ILDAGMFFAKRTG---PHVDYNPQPAIDYLRT-- 332
Gp33  Che8    LLDAGMFFAKRTG---PHVDYNVQPAIDYLRT-- 333
Gp70  Corndog LIDAGMFFAKGTG---PHVDYGIGPAIEYLRS-- 346
Gp238 Bxz1    FMDAGLFFVKGTR---PHTNYHIGAAVDYLRS-- 342
Gp9   Bxb1    VLDAGLFFIRGTG---PHVNYNIDPATDFLRSVT 322
Gp52  Wildcat ISDAGMFFINRTT---PHINYNVGPAVDFLAGL- 321
Gp12  L5      IIDALTFFAKSTQGPSWPHLYNRFPAVEFLRRI- 254
Gp12  Bxz2    IIDTLRFFTNTAHG------YNIGPAIDFLRS-- 321
              . *:  ** .          *   .* ::*
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Figure 2. CLUSTALW alignment of Ms6 LysB deduced amino acid sequence and mycobacteriophage putative LysB protein sequences. Mycobacteriophages: Che8 Gp33 (Q855H7), Corndog Gp70 (Q856M4), Bxz1 Gp238 (Q852W7), Bxb1 Gp9 (Q9B0B2), Wildcat Gp52 (Q19Y08), L5 Gp12 (Q05328) and Bxz2 Gp12 (Q857L1); the primary accession numbers of the UniProtKB/TrEMBL database are given in parentheses. Identical (*), highly similar (:) and similar (.) amino acids are indicated. Dashes represent gaps introduced by CLUSTAL to optimize the alignment. The conserved Asp and His residues are indicated in bold. The conserved pentapeptide is highlighted on a grey background. Numbers refer to the amino acid positions.

Enzymic activity of LysB

In order to determine if His6-LysB had lipolytic activity as expected, its hydrolytic capacity was tested using LB agar plates containing several substrates. The His$_6$-LysB spot produced a zone of clearance when incubated on esterase indicator plates containing the short-chain acylglycerol tributyrin. A zone of a white precipitate was also observed in plates containing Tween 80 or Tween 20 (Fig. 3). Tween 80 and Tween 20 are esters of oleic (C$_{18}$) and lauric (C$_{12}$) acids, respectively, and can be cleaved by lipolytic enzymes to produce a fatty acid and an alcohol. The presence of Ca^{2+} causes the formation of an insoluble fatty acid salt, which was seen as a white precipitate.

On true lipase indicator plates (triolein plus rhodamine B), enzymic activity of His$_6$-LysB was also observed. Rhodamine B formed a fluorescent complex with the free fatty acids released from the hydrolysis of lipids, and this was seen under UV light (Kouker & Jaeger, 1987).

Fluorescence was observed after an incubation period of 24 h, and it became more intense after an additional 24 h incubation period (Fig. 3). This result demonstrates that His$_6$-LysB has lipolytic activity on lipase substrates. No activity was observed with the LysB buffer (PBS) used as a negative control. Specific and sensitive detection of lipase activity can be achieved by the spectrophotometric detection of resorufin released from the artificial triglyceride 1,2-Odilauryl-*rac*-glycero-3-glutaric acid resorufin ester (Jaeger *et al.* 1999; Schmidt *et al.*, 2004).

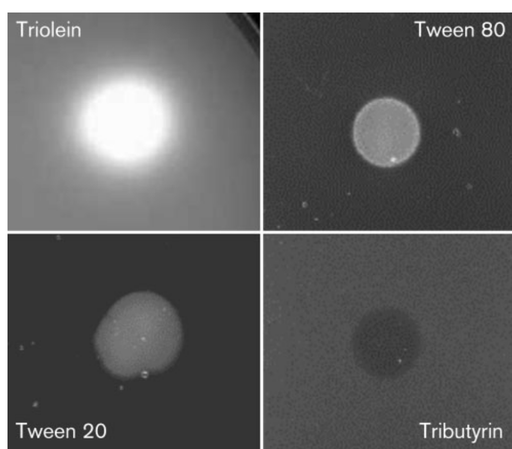

Figure 3. Effect of His$_6$-LysB activity on agar plates containing triolein and rhodamine B, tributyrin, Tween 20 and $CaCl_2$, and Tween 80 and $CaCl_2$. The plates were incubated with 100 µg purified His$_6$-LysB for at least 24 h at 37 °C.

As can be observed in Fig. 4, His$_6$-LysB was able to hydrolyse this substrate releasing resorufin (Fig. 4) with a ΔA_{572} of approximately 0.14 min^{-1}, confirming that His$_6$-LysB has lipase activity.

The enzymic activity of recombinant His$_6$-LysB was further examined on agar plates containing skim milk or sheep blood erythrocytes. No zones of clearance were observed following incubation for more than 120 h (data not shown), indicating that His$_6$-LysB does not have proteolytic or haemolytic activity.

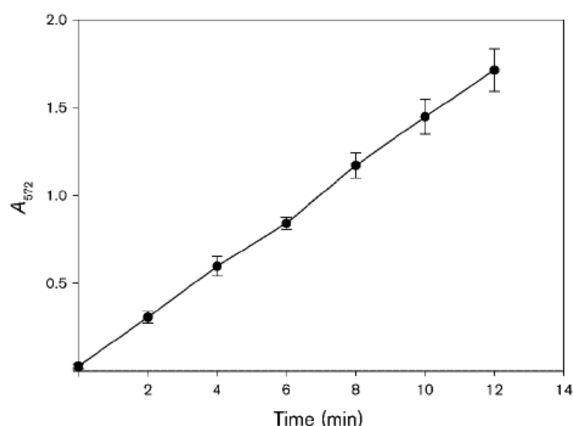

Figure 4. Kinetic recordings of the hydrolysis of resorufin ester by recombinant LysB at pH 7.5. The release of resorufin was measured by absorbance readings at 572 nm. The values shown are means from three independent determinations, with error bars (SD) indicated.

Biochemical characterization of LysB lipase

The activity of His_6-LysB was measured using pNP esters of different chain lengths. As shown in Fig. 5, His_6-LysB was able to hydrolyse all the tested substrates, showing the highest activity (0.12 U mg^{-1}) with the short-chain pNPB (C_4). In order to examine the specificity of His_6-LysB towards the length of the acyl chain, the kinetic parameters of the enzyme were determined.

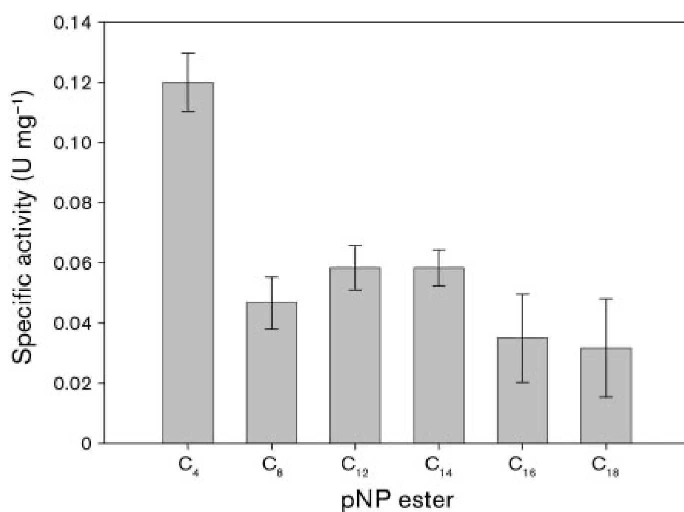

Figure 5. Lipolytic activity of LysB towards various pNP esters (as measured spectrophotometrically). The values shown are means from three independent determinations, with errors bars (SD) indicated. One unit (U) of enzyme activity corresponds to the liberation of 1 µmol p-NP min^{-1}.

The rates of hydrolysis of different concentrations of all substrates were measured, and the substrate affinity constant ($K_{0.5}$) and the turnover of the enzymic reaction (K_{cat}) were obtained, and are shown with the deduced catalytic efficiency ($K_{cat}/K_{0.5}$) (Table II). The $K_{0.5}$ values had a tendency to decrease with the increase in the chain length of substrates. The shortchain pNPB showed the highest K_{cat} and $K_{0.5}$ values, resulting in a low catalytic efficiency (0.61 min^{-1} μM^{-1}). Although the highest affinity was observed for the longchain pNPP and pNPS, 145 µM and 146 µM respectively, pNPS presented a lower catalytic efficiency (0.80 min^{-1} μM^{-1}) than pNPP (1.07 min^{-1} μM^{-1}).

Table II. Kinetic parameters of recombinant LysB for the hydrolysis of pNP esters of different chain lengths (C_4–C_{18}) as substrates

pNP ester	K_{cat} (min^{-1})*	$K_{0.5}$ (μM)*	$K_{cat}/K_{0.5}$ (min^{-1} μM^{-1})
pNPB (C_4)	449 ± 26	726 ± 24	0.61
pNPC (C_8)	247 ± 20	348 ± 1	0.71
pNPL (C_{12})	297 ± 38	260 ± 30	1.14
pNPM (C_{14})	278 ± 32	246 ± 3	1.13
pNPP (C_{16})	146 ± 0.32	145 ± 8	1.07
pNPS (C_{18})	117 ± 3.2	146 ± 34	0.80

*Values are means ± SD from three independent determinations.

The highest catalytic efficiency was observed with the relatively long-chain-length pNPL and pNPM, with catalytic efficiencies of 1.14 and 1.13 min^{-1} μM^{-1}, respectively, indicating that these are the best substrates among the pNP esters examined.

Effect of temperature and pH on LysB activity

The effect of temperature and pH on the activity of His6-LysB was determined using pNPB as the substrate. Although a high level of residual activity (72 %) was observed in the tested temperature range (4–63 °C), the highest specific activity was achieved at 23 °C (Fig. 6a). The enzyme shows maximal activity at pH 7.5–8.8, maintaining an activity above 50% in the pH range 6.8–9.5. Almost no activity was observed at pH 5 (2.2 %) (Fig. 6b).

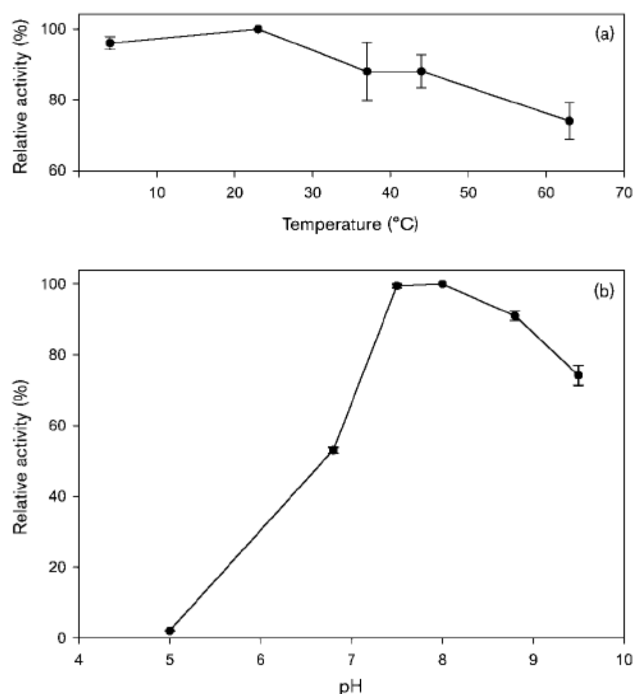

Figure 6. Effect of temperature (A) and pH (B) on activity of His6-LysB using pNPB as the substrate. The values represent relative activity (%), taking the highest value of His6-LysB activity as 100 %. The values shown are means from three independent determinations, with errors bars (SD) indicated.

Effect of different metal ions and inhibitors

The effect of different metal cations on the activity of His_6-LysB was assayed using pNPB as the substrate. Table 2 indicates that the presence of Ca^{2+} or Mn^{2+} increased the activity of the recombinant protein; this was seen especially with Ca^{2+} (5 mM), which increased the activity by 99.2 %. Mg^{2+} and K^{+} made no significant difference on the activity of the enzyme, while Zn^{2+}, Cd^{2+} and Hg^{2+} caused a reduction in His_6-LysB activity (Table 2), possibly by destabilizing the active conformation of the enzyme. In the presence of EDTA, no significant change in activity was observed, indicating that the enzyme is not a metallohydrolase. Complete inhibition was obtained with 10 mM PMSF, supporting the notion that a Ser residue is part of the catalytic triad (Table III).

Table III. Effect of different metal ions and inhibitors on His6-LysB activity using pNPB as the substrate Metal ions and inhibitors were incubated with recombinant LysB in the assay buffer for 2 h at 23 °C before the assay for activity.

Compound	Concentration (mM)	Relative activity (%)*
Ca^{2+}	5	199.2 ± 2.5
Mg^{2+}	5	108.8 ± 0.9
K^{+}	5	99.2 ± 2.0
Cd^{2+}	5	3.4 ± 0.8
Hg^{2+}	5	6.5 ± 0.4
Mn^{2+}	5	136.1 ± 0.9
Zn^{2+}	5	6.2 ± 0.5
EDTA	10	114.4 ± 3.6
PMSF	10	0.0 ± 0.0
DEPC	20	54.1 ± 0.3

*The enzyme activity of recombinant LysB without the addition of ions or inhibitors was taken to be 100 %. Values are means ± SD from three independent determinations.

The presence of DEPC at a concentration of 20 mM reduced the activity to 54% (Table 2), suggesting the possibility that His residues are also involved in catalysis.

Ser168, Asp215 and H246 form the catalytic triad

As stated above, the amino acids Ser168, Asp215 and His246 are good candidates for the catalytic triad (Fig. 2). To determine the involvement of these amino acids in the enzymic activity, mutant proteins were generated by changing Ser168, Asp215 or His246 to Alanine. Activity of the purified proteins was measured using pNPB as a substrate, as described previously. As expected due to the position in the Gly-X-Ser-X-Gly motif, our results confirmed that Ser168 is part of the catalytic triad since the catalytic efficiency of S168A was reduced to only 4.57% (Fig. 7). Our results also indicate that Asp215 and His246 are also part of the

catalytic triad, since their exchange for an Alanine resulted in a loss of catalytic efficiency of 86.17% and 91.49%, respectively. Although Asp306 was also conserved among the aligned LysB proteins, the D306A mutant was still active showing a catalytic efficiency of 76.59%.

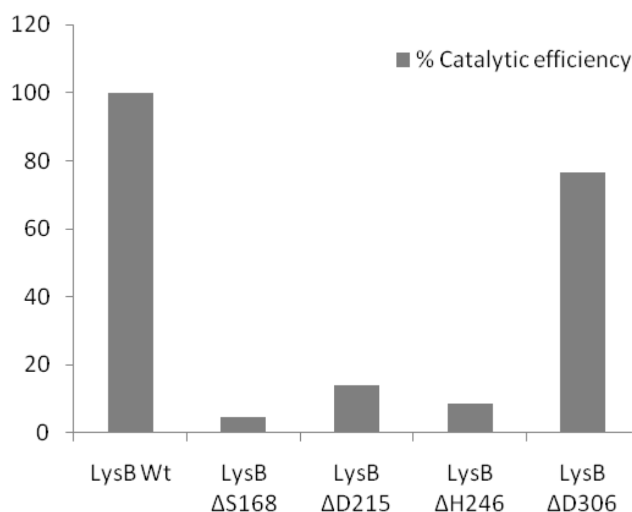

Figure 7 Catalytic efficiency of wild type LysB and mutant proteins.

Discussion

In the present work, we described a gene, within the lytic cassette of the mycobacteriophage Ms6, encoding an enzyme with lipolytic activity. The gene lysB, which is localized downstream of the endolysin gene *lys*A, and immediately upstream of the holin gene *hol*, is 996 bp in length, and encodes a 332 aa protein (Garcia *et al.*, 2002). The presence of the pentapeptide Gly-Tyr-Ser-Gln-Gly within the amino acid sequence matches the consensus motif Gly-X-Ser-X-Gly characteristic of lipolytic enzymes, and led us to search for lipolytic activity. Since lipolytic enzymes include carboxylesterases and true lipases (Arpigny & Jaeger, 1999), activity of the recombinant protein was tested on esterase and lipase substrates. His_6-LysB showed esterase and lipase activity on agar plates containing Tween 20, Tween 80, tributyrin or triolein. Definitive evidence of the lipase activity was subsequently demonstrated by the released resorufin from the artificial triglyceride 1,2-Odilauryl-*rac*-glycero-3-glutaric acid resorufin ester (Fig. 4).

Lipolytic enzymes are also characterized by their ability to catalyse the hydrolysis of a wide range of fatty acid esters. Lipase and esterase activity may be distinguished by their substrate specificity using pNP esters (Bornscheuer, 2002). Although His_6-LysB displayed activity

towards substrates with chain lengths from C_4 to C_{18}, it showed a higher affinity for pNP esters of longer chain length (C_{16} and C_{18}). These results, together with the higher catalytic efficiency revealed for C_{12} and C_{14} pNP esters, indicate that the longchain substrates must represent the natural substrate of His$_6$-LysB, and therefore this enzyme could be classified as a lipase. However the amino acid sequence of LysB does not show characteristic features of any of the lipase families identified by Arpigny & Jaeger (1999). Cutinases, which are also lipolytic enzymes, can be considered as a link between esterases and lipases because they are able to efficiently hydrolyse soluble esters and emulsified triacylglycerols (Mannese *et al.*, 1995; Chen *et al.*, 2007; Longhi & Cambillau, 1999; Carvalho *et al.*, 1999). Although the LysB pentapetide (Gly-Tyr-Ser-Gln-Gly) is exactly the same as that in most cutinases described so far, the amino acid sequence around the conserved motif does not match the PROSITE (www.expasy.org/prosite/) pattern of cutinases. At this point, we cannot clearly classify LysB as a lipase or a cutinase. Although no sequence similarity was observed with any of the known lipases or with proteins belonging to the cutinase family, a high degree of identity was observed with the deduced amino acid sequences of proteins with unknown function encoded within the lysis cassette of mycobacteriophages infecting the non-pathogenic strain *M. smegmatis* or the pathogenic *Mycobacterium tuberculosis*. Alignment of these proteins showed that the characteristic pentapetide Gly-Tyr-Ser-Gln-Gly is very well conserved among these proteins. Along with this consensus motif, conserved Asp and His residues were also identified, suggesting that some of them may be involved in the catalytic triad. The complete inhibition of His$_6$-LysB by PMSF indicated that the inhibitor might be bound to the nucleophilic Ser residue at the highly conserved catalytic triad, and that this Ser residue may be easily accessible to the substrate. This result leads us to conclude that LysB belongs to the family of serine hydrolases, as is the case for lipid hydrolases (Holmquist, 2000). In the presence of the His inhibitor DEPC, His$_6$-LysB activity decreased 46 %, indicating that His residues participate in the active site of the enzyme. Inhibition by such compounds has been described for enzymes involving Ser and His residues in their active site (Teo *et al.*, 2003; Nawani *et al.*, 2006). We suggest that His246 is the Histidine residue involved in catalysis since activity of the H246A mutant enzyme was reduced to 8.51%. Although three Asp residues, Asp215, Asp249 (we were not able to obtain this mutant) and Asp306 on LysB amino acid sequence are totally conserved. The D215A mutant showed a loss of activity of 86.17% indicating that Asp215 together with His246 are in fact the amino acids involved in the catalytic triad obeying the order Ser-Asp-His. In addition to these conserved residues, blocks of amino acids are also conserved among the mycobacteriophage putative lipolytic enzymes, and these are different from members of the eight lipase families described by Arpigny & Jaeger (1999). Hence, LysB and its mycobacteriophage putative proteins appear to be part of a novel family of lipolytic enzymes. His$_6$-LysB was active over a wide temperature range, showing optimal activity at 23 °C and pH

7.5–8 (Fig. 6). Activity over a wide range of temperatures and pH has been described for other lipolytic enzymes (Kaiser *et al.*, 2006; Teo *et al.*, 2003). Although the optimum pH may vary substantially, most serine hydrolases show little or no activity below pH 5 (Schmidt *et al.*, 2004), as was observed with His_6-LysB. The activity of the enzyme was affected by the presence of metal ions. It is possible that Zn^{2+}, Cd^{2+} and Hg^{2+} affect the catalytic site directly, since incubation of the recombinant His_6-LysB with these cations resulted in a severe loss of activity (Table 2). A similar effect of these ions on the activity of microbial lipases has been observed by other authors (Kaiser *et al.*, 2006; Nawani *et al.*, 2006). Hg^{2+} is a thiol-reactive agent, and its ability to compromise His_6-LysB activity suggests that thiol groups may be important for catalysis (Teo *et al.*, 2003). In contrast, Ca^{2+} and Mn^{2+} exhibited a stimulatory effect on the enzyme activity. Ca^{2+} has been shown to increase the lipolytic activity of several enzymes, and is known to function in the structural stabilization and activation of those enzymes (Arpigny & Jaeger, 1999; Ma *et al.*, 2005; Kaiser *et al.*, 2006). Hence, we can improve the activity of the recombinant lipase by adding selective metal ions to the reaction system.

The results presented in this work clearly demonstrate that LysB is a lipolytic enzyme. One can question what would be the role of a lipase in cell lysis. An important feature must be remembered: unlike other Gram-positive bacteria, mycobacteria have a complex cell wall with a high content of lipids (over 60 %). They possess a cell envelope structure based on long-chain mycolic acids esterified to an arabinogalactan polysaccharide, which is attached to the peptidoglycan backbone. This mycolyl–arabinogalactan–peptidoglycan complex (core) intercalates with an array of unusual free lipids, resulting in an effective external permeability barrier (Brennan, 2003; Minnikin, 1982). As happens with bacteriophages that infect Gram-negative hosts and contain additional lysis genes, such as the genes encoding Rz and Rz1 (Young, 2005; Summer *et al.*, 2007), bacteriophages infecting mycobacteria may also need additional lysis genes. Due to the complexity of the cell wall of mycobacteria, it is easy to understand why a mycobacteriophage would encode a lipolytic enzyme, since one of the principles of phage lysis mentioned by Ry Young is that 'the lysis process, once initiated, should be rapid in order to improve phage production' (Young, 2005).

Our overall results open the research into other cell wall hydrolases encoded by mycobacteriophages that target one of the most important human pathogens, *M. tuberculosis*, for which new therapeutic alternatives are urgently needed.

3

Mycobacteriophage Ms6 LysB specifically targets the outer membrane of *Mycobacterium smegmatis*

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Summary

LysB, a mycobacteriophage Ms6-encoded protein, was previously identified as a lipolytic enzyme able to hydrolyse the ester bond in lipase and esterase substrates. In the present work, we show that LysB can hydrolyse lipids containing mycolic acids from the outer membrane of the mycobacterial cell wall. LysB was shown to hydrolyse the mycolic acids from the mycolylarabinogalactan–peptidoglycan complex where the mycolates of the inner leaflet of the outer membrane are covalently attached to an arabinosyl head group. In addition, treatment of the extractable lipids from *Mycobacterium smegmatis*, *Mycobacterium bovis* BCG and *Mycobacterium tuberculosis* H37Ra with LysB showed that trehalose 6,6'-dimycolate (TDM), a trehalose diester of two mycolic acid molecules, was hydrolysed by the enzyme. We have also determined the structures of the mycolic acid molecules that form the *M. smegmatis* TDM. The identification of a phage-encoded enzyme that targets the outer membrane of the mycobacterial cell wall enhances our understanding of the mechanism of mycobacteriophage lysis.

Introduction

We have been undertaking bacteriophage studies with the aim of exploring their ability to destroy their host as a consequence of their lytic cycle. These studies are driven by either the use of bacteriophage itself as a therapeutic agent (phage therapy) or exploring the enzymes involved in cell lysis for enzyme therapy against pathogenic bacteria. At the end of their virulent cycle, all double-stranded DNA phages produce at least two enzymes: an endolysin, which hydrolyses the peptidoglycan, and a holin, a small membrane protein that defines the time of lysis and allows the phage endolysin to cleave its target. This leads to cell lysis and release of the progeny phage (Young *et al.*, 2000; Young, 2005). Endolysins are also capable of degrading peptidoglycan when applied externally to the bacterial cell wall, resulting in a rapid lysis of the bacterial cell (Fischetti, 2005). These enzymes are normally very specific to the bacterial host of the phage from which they were derived (Fischetti, 2001) and this capacity renders them promising potential antibacterial agents. The use of purified bacteriophage lysins to destroy their bacterial hosts has been the subject of several recent reports (Loeffler *et al.*, 2001; Schuch *et al.*, 2002; Yoong *et al.*, 2004; Zimmer *et al.*, 2002). The data reported so far are all on Gram-positive bacteria, since direct contact of endolysins with the peptidoglycan is only possible in such bacteria; in Gram-negative bacteria, the presence of an outer membrane prevents this direct interaction. In addition to endolysins and holins, some bacteriophages encode additional genes within their lytic cassettes. Bacteriophage lambda encodes the Rz and Rz1 proteins. These proteins were recently described as being involved in the fusion of the inner and outer membranes, which thus eliminates all the conceivable barriers to the release of the progeny virions (Berry *et al.*, 2008). Genes encoding similar gene products were identified in several other phages infecting Gram-negative hosts (Summer *et al.*, 2007). Thus, our present knowledge of the mechanism of bacteriophage lysis suggests that the complexity of phage lytic cassettes depends on their hosts. Hosts with a simpler envelope require the phage to possess a simple lytic cassette, whereas hosts with a complex envelope require the phage to have a more complex lytic cassette.

In mycobacteriophage Ms6, a temperate phage that infects *Mycobacterium smegmatis* (Portugal *et al.*, 1989), the lytic cassette comprises five genes (Garcia *et al.*, 2002). In addition to the holin and the endolysin, Ms6 codes for a protein known as LysB. LysB was recently identified as a protein with lipolytic activity showing a higher affinity for substrates with long chain lengths (Gil *et al.*, 2008).

Despite being classified as Gram-positive bacteria, mycobacteria have a complex cell wall. This cell wall consists of peptidoglycan covalently linked to arabinogalactan, which is in turn

esterified to a variety of long chain (C_{60}–C_{90}), α-branched, β-hydroxy fatty acids (mycolic acids). This covalently linked complex, called mycolyl-arabinogalactan peptidoglycan (mAGP), is known as the cell wall core and forms the stable scaffolding to anchor the outer noncovalently associated lipids and glycolipids, including trehalose 6,6'-dimycolate (TDM or cord factor) (Daffé & Draper, 1998; Daffé, 2008). TDM, probably the most prominent and best-studied mycolic-acid-containing compound of mycobacteria, is composed of a trehalose molecule esterified with two mycolic acids and is implicated in the pathogenesis of certain mycobacterial species (Rao *et al.*, 2005; Fujita *et al.*, 2005). The mycolyl arabinogalactan together with TDM and trehalose 6'-monomycolate is responsible for the highly hydrophobic cell surface properties and acid-fastness of the mycobacterial cells, resulting in a resistance to dehydration and a natural impermeability to nutrients and antibacterial drugs (Barry *et al.*, 1998; Crick & Brennan, 2008, Liu *et al.*, 1996).

Given the complexity of the mycobacterial cell envelope, it is easy to understand why mycobacteriophages encode an additional lysis enzyme (LysB) that hydrolyses long chain esters (Gil *et al.*, 2008). In the present work, we identify the substrates for Ms6 LysB activity in the cell envelope of mycobacteria. Thus, we provide evidence that Ms6 LysB targets the *M. smegmatis* outer membrane by cleaving the ester bond between mycolic acids and arabinogalactan in the mAGP complex and between mycolic acids and trehalose in TDM. Moreover, its activity is not species-specific, as Ms6 LysB could also act on the TDM from *Mycobacterium tuberculosis* H37Ra and *Mycobacterium bovis* BCG.

Methods

Plasmids, bacterial strains and culture media

Plasmid pMP302, which encodes LysB, has been described previously (Gil *et al.*, 2008). *Escherichia coli* JM109 (pMP302) was grown at 37 °C in Luria–Bertani broth or agar containing 100 µg ampicillin ml^{-1}. *M. smegmatis* mc^2155 (Snapper *et al.*, 1990), *M. bovis* BCG (Pasteur strain ATCC 35734) and *M. tuberculosis* H37Ra (ATCC 25177) were grown in Middlebrook 7H9 (supplemented with 0.05% Tween 80, 0.2% glycerol) at 37 °C. For the growth of *M. bovis* BCG and *M. tuberculosis* H37Ra, the medium was also supplemented with 10% oleic acid/albumin/dextrose/catalase enrichment (BD Biosciences).

Chemical and lipid standards.

The TDM from *M. tuberculosis* (MTb TDM) and the trehalose standards were purchased from Sigma. All solvents were HPLC-grade and were purchased from Fisher Scientific.

Expression and purification of LysB protein

6-His-tagged LysB was expressed and purified from *E. coli* JM109 (pMP302) as described previously (Gil *et al.*, 2008).

Preparation of M. smegmatis cell wall components

mAGP from *M. smegmatis* was obtained as described previously (Bhamidi *et al.*, 2008). In addition, the purified mAGP was extracted with diethyl ether at room temperature, overnight, in order to remove all the free mycolic acids. The mixture was centrifuged at 2500 *g* for 5 min and the pellet was washed once more with diethyl ether. The pellet was dried under nitrogen and left in a chemical hood for a couple of hours.

For total lipid extraction, the bacterial culture was centrifuged after growing for 24 h and the wet cell mass was extracted with chloroform/methanol [2:1, v/v, 15 ml (g wet mass)$^{-1}$] at 55 °C overnight. The extracts were evaporated by rotary evaporation. The dried crude extract was stored at room temperature for further use.

Treatment of mAGP with LysB

Purified mAGP (1 mg) was treated with LysB (360 µg) in PBS pH 7.4, containing 0.2% Triton X-100 in a total volume of 400 µl. The mAGP was suspended using an ultrasonic water bath for 1 min and the incubation was continued for 24 h at room temperature with shaking. The mycolic acids were extracted with 1 ml diethyl ether and washed with water. The organic and water layers were separated by centrifugation and the organic phase (upper layer) was collected and washed once with 1 ml water. After drying, the samples were analysed by liquid chromatographymass spectrometry (LC-MS) in negative ion mode.

To test the effect of LysB on mycobacterium lipids, 200 µg total lipids (extracted from *M. smegmatis*, *M. bovis* BCG or *M. tuberculosis* H37Ra) were dried in a glass tube and resuspended in PBS buffer using an ultrasonic water bath. LysB (360 µg) was added and the samples were incubated at 37 °C for 24 h. After treatment, chloroform and methanol were added to the samples such that the ratio was 8:4:3 (chloroform/methanol/water); the organic layer was then transferred to a new tube and dried. For analysis, total lipids were redissolved in

chloroform to 10 mg ml^{-1} and then separated by 1D thin-layer chromatography (TLC) on aluminium-backed silica 60 F254 gel plates (EMD Chemicals) developed with chloroform/methanol/water (20:4:0.5; by volume) and visualized with 10% $CuSO_4$ in 8% H_3PO_4 followed by charring at 200 °C. In parallel, control assays were performed in the same conditions using PBS instead of the Ms6 LysB protein.

Separation and purification of the lipid hydrolysed from M. smegmatis by LysB (Msmeg_1)

The crude lipid extract (20 mg) was applied to a Silica Gel 60 (Merck, 70–230 mesh) column (2 x 50 cm) and eluted successively with chloroform (4 x 100 ml), acetone (4 x 100 ml) and methanol (4 x 100 ml). Fractions of 100 ml were collected, dried and monitored for Msmeg_1 by TLC.

Determination of Msmeg_1 structure

Purified Msmeg_1 was analysed for the presence of mycolic acids as described by Phetsuksiri *et al.* (1999). Briefly, the lipid was resuspended in 2 ml of 15% tetrabutylammonium hydroxide (TBAH; Sigma) and saponified at 100 °C overnight. After cooling, 2 ml water, 3 ml dichloromethane and 300 µl iodomethane (Aldrich) were added to the entire reaction mixture, which was then left on a shaking platform for 4 h. After centrifugation, the upper layer was discarded and the lower organic phase was washed three times with 3 ml water. The washed lower phase was dried by nitrogen flow, extracted with 4 ml diethyl ether, sonicated for 5 min and centrifuged at 2500 *g*. The ether extract was transferred into new glass tubes and dried. The mycolic acid methyl esters (MAMEs) from intact *M. smegmatis* were obtained in a similar fashion, starting with whole cells. MAMEs derived from Msmeg_1 and from *M. smegmatis* were fully separated into subclasses by threefold TLC developed with *n*-hexane/ethyl acetate (95:5; volume/volume) and then silica scrapings were extracted in diethyl ether overnight. The ether extracts were washed with water, dried and analysed by LC-MS. The mycolic acid methyl esters were visualized with 10% $CuSO_4$ in 8% H_3PO_4 spray followed by charring at 200 °C for analytical purposes.

In order to determine the sugar composition of Msmeg_1, alkaline hydrolysis was performed on TDM standard and pure Msmeg_1 (10 µg of each sample) by adding 1 ml of 1 M KOH/methanol for 2 h at 80 °C. Extracts were cooled to room temperature and neutralized with glacial acetic acid. The sugars were extracted with chloroform/water (1:1, v/v) and the aqueous layer containing the sugars was dried under nitrogen flow. The samples together with the trehalose standard (1 µg), to which was added pyridine, were acetylated with 100 µl of acetic anhydride (Sigma) at 120 °C for 1 h. The extracts were cooled at room temperature and

extracted with chloroform/water (1:1, v/v). The resulting acetylated products were analyzed by GC-MS with Varian CP-3800 Gas Chromatograph (Varian, CA, USA), using a FactorFour Capillary Column (VF-5ms, 30m x 0.25mm, 0.25 μm I.D., Varian). GC oven was operated at 50 °C for 1 min, and then the temperature was increased to 150 °C at a rate of 30 °C/min. From 4.33min to 36.8min, the temperature was increased to 330 °C at a rate of 8 °C/min. The retention times and mass spectra were compared with those of trehalose standard.

LC-MS assay

LC-MS was performed on an Agilent 6220 high resolution time of flight mass spectrometer equipped with a MultiMode source selected to be in the dual atmospheric pressure chemical ionization/electrospray ionization mode. The HPLC is equipped with an Agilent 1200 binary pump. In LC-MS experiments, a Waters XBridge (C18, 2.1 x 150 mm) with 5 μM particle size was used. For analysis of free mycolates (negative mode), mycolate esters (positive mode) and intact TDM (positive mode), the same HPLC conditions were used. The column temperature was 45 °C. The separation was done using a gradient of solvent A (99% methanol, 1% 500 mM ammonium acetate) and solvent B (79% *n*-propanol, 20% hexane, 1% 500 mM ammonium acetate) from 100% A to 100% B at a flow rate of 0.32 ml min^{-1} over a period of 45 min. The drying gas temperature was 300 °C and the vaporizer temperature was set at 200 °C. Typically, 5 μl of a 1 mg ml^{-1} sample was injected for analysis. The mass spectrum was acquired from *m/z* 250 to 3200 Da with a frequency of one scan per second. The resulting data were analysed by using Agilent's Mass Hunter program.

Results and Discussion

LysB hydrolyses the mAGP complex

In order to evaluate the ability of LysB to act on mAGP, this complex was obtained from *M. smegmatis* and incubated with LysB for 24 h. Released mycolic acids were extracted and analysed by LC-MS (negative ion mode). As shown in Fig. 1, free mycolic acids were observed in samples treated with LysB, while in the control sample (where LysB is absent) no mycolic acids were detected. The dominant mycolic acids were those expected from *M. smegmatis* – α- and α'-mycolates – which contain no oxygen beyond the hydroxyl group in the meromycolate moiety, as well as epoxy mycolates containing additional oxygen in the meromycolate moiety

(Barry *et al.*, 1998; Marrakchi *et al.*, 2008). Since, in the mAGP complex, the non-reducing termini pentaarabinosyl motifs in the arabinogalactan are esterified to mycolic acids, the observation of the release of mycolic residues demonstrates that LysB acts on this bond and thus disrupts the inner leaflet of the mycobacterial outer membrane (Hoffmann *et al.*, 2008; Zuber *et al.*, 2008). This result is consistent with the esterase activity of Ms6 LysB, previously reported by our group (Gil *et al.*, 2008), and with the report of a mycolyl arabinogalactan esterase activity for the homologous LysB found in mycobacteriophage D29 (Payne *et al.*, 2009).

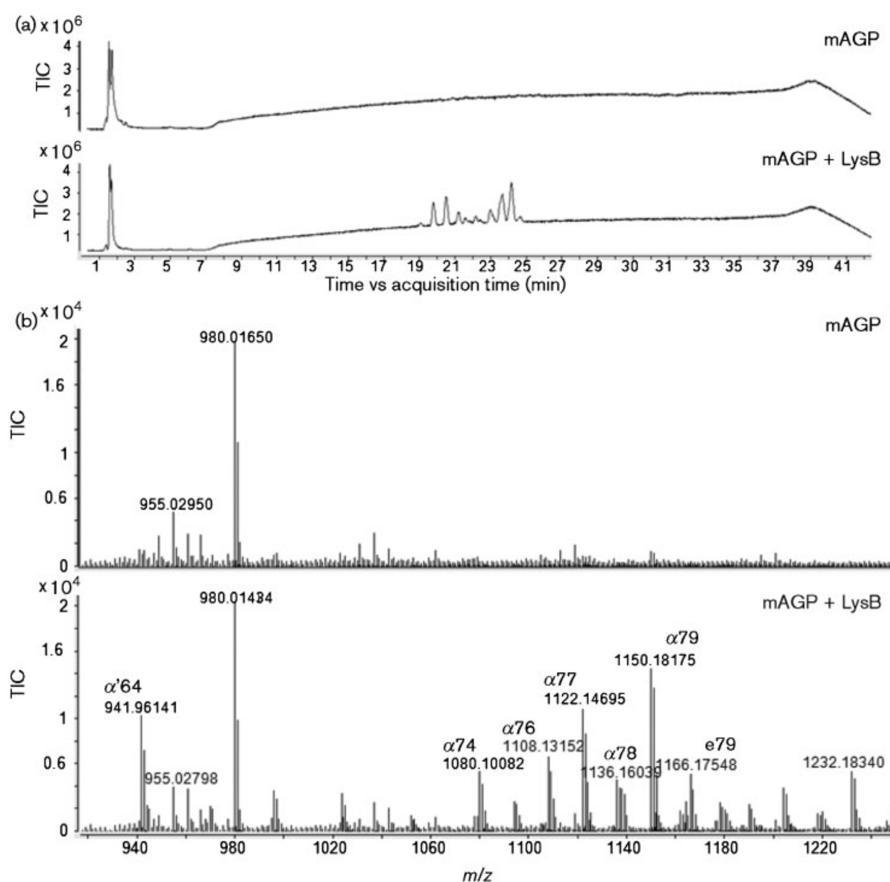

Figure 1. LC-MS (negative ion mode) identification of mycolic acids released from mAGP after LysB treatment. (a) Total ion chromatograms from non-treated and treated (LysB) mAGP samples. (b) The negative ion mass spectra (averaged from 19 to 25 min) of non-treated and treated mAGP samples from *m/z* 920 to 1232. The mAGP sample treated with LysB showed the presence of several peaks, identified as mycolic acids. α, α-mycolates; α’, α’-mycolates; e, epoxy mycolates. The ion at *m/z* 980 corresponds to a calibration reference compound.

Despite the fact that these homologous proteins share a low identity with the amino acid sequence, they have in common the conserved motif GXSXG of lipolytic enzymes (Gil *et al.*, 2008, Hatfull *et al.*, 2006). This suggests a common function and thus a common substrate for them. The esterase activity against the mycobacterial outer membrane is parallel to the lambda Rz/Rz1 homologues of Gram-negative bacteria, which, as proposed by Berry *et al.* (2008), mediate the final step of host lysis by fusing the inner and outer membranes. Although different from a Gram-negative outer membrane, the existence of an outer membrane layer in the

mycobacterial envelope has long been postulated (Minnikin, 1982). Despite the fact that its arrangement is still under debate, recent work has clearly shown its existence (Hoffmann *et al.*, 2008; Zuber *et al.*, 2008). The existence of additional lysis genes in mycobacteriophage genomes, coding for proteins able to break the outer membrane linkage to the cell wall skeleton, enables the mycobacterial phage to efficiently escape from their lysed host.

LysB hydrolyses lipids from the extractable lipid fraction

Taking into account that LysB has a broad range of lipolytic activity (Gil *et al.*, 2008), we decided to investigate its ability to hydrolyse other cell envelope lipids. Total extractable lipids from *M. smegmatis* were extracted and treated with LysB. TLC analysis revealed that the lipid profile changes after LysB treatment (Fig. 2, lanes 1 and 2). One of the major lipids disappears from the LysB-treated sample. Interestingly the same result is observed with total lipids from *M. tuberculosis* H37Ra (Fig. 2, lanes 4 and 5) and *M. bovis* BCG (data not shown). This indicates that LysB is not specific for *M. smegmatis*, as it is able to hydrolyse lipids from other mycobacterial species. The lipid hydrolysed from *M. smegmatis* by LysB was designated Msmeg_1. Its R_F value was very similar to that of the *Mycobacterium tuberculosis* (MTb) TDM (Fig. 2, lane 3), suggesting that it could be the *M. smegmatis* version of TDM. The long acyl chains of the mycolic acid residues differ in structure and chain length within TDM and thus, TDMs from different mycobacteria have slightly different R_F values (Fujita *et al.*, 2005).

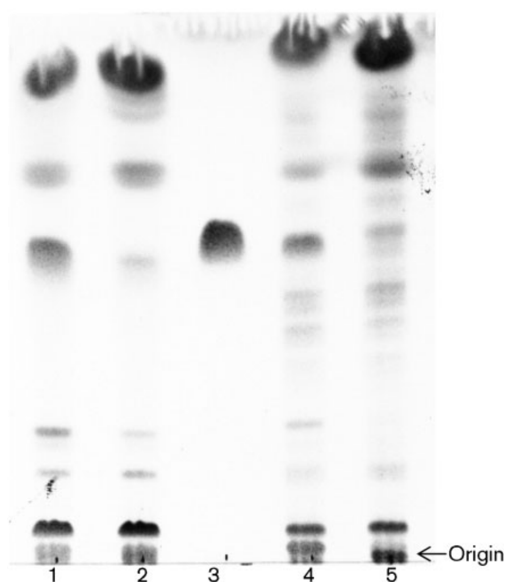

Figure 2. Thin layer chromatogram developed with chloroform/methanol/water (20: 4:0.5 by vol.) of total lipid extract from *M. smegmatis* (lane 1), total lipid extract from *M. smegmatis* treated with LysB (2), Mtb TDM standard (3), total lipid extract from M. tuberculosis H37Ra (4) and total lipid extract from *M. tuberculosis* H37Ra treated with LysB (5).

Msmeg_1 is the *M. smegmatis* trehalose dimycolate

To investigate the composition of Msmeg_1, we produced a large amount of *M. smegmatis* cell mass, extracted Msmeg_1 and purified it by silica gel chromatography. To search for the presence of mycolic acids, Msmeg_1 was de-esterified with TBAH and concomitantly, the released fatty acids were methyl esterified with methyl iodide and then analysed by using TLC. After a threefold development with *n*-hexane/ethyl acetate (95:5, v/v), we observed three groups of MAMEs from both Msmeg_1 and intact *M. smegmatis* (Fig. 3a). The three spots observed correspond to α-, α'- and epoxy mycolates. The different types of MAMEs were eluted from the TLC and analysed by LC-MS. This analysis revealed the presence of M + Na ions with *m/z* values ranging from 951 to 1274, and the major components were identified as shown in Fig. 3(b). This result demonstrates that Msmeg_1 has mycolic acids in its structure. The pseudo-molecular ions obtained show a mass increase of 38 amu compared with those in Fig. 1. This corresponds to the mycolic acids being analysed as methyl esters and thus detected as sodiated methyl esters (positive mode) rather than as non-esterified carboxylate anions (negative mode). In addition, after separation into the mycolate classes, more details can be seen than are apparent in Fig. 1, namely the epoxy mycolates range from 74 to 84 carbon atoms, the α'-mycolates range from 62 to 66 carbon atoms and the α-mycolates range from 74 to 82 carbon atoms. As is the case for the free mycolates (Fig. 1), the exact mass values for components identified as epoxy mycolates are all consistent with the additional oxygen atom in the molecule.

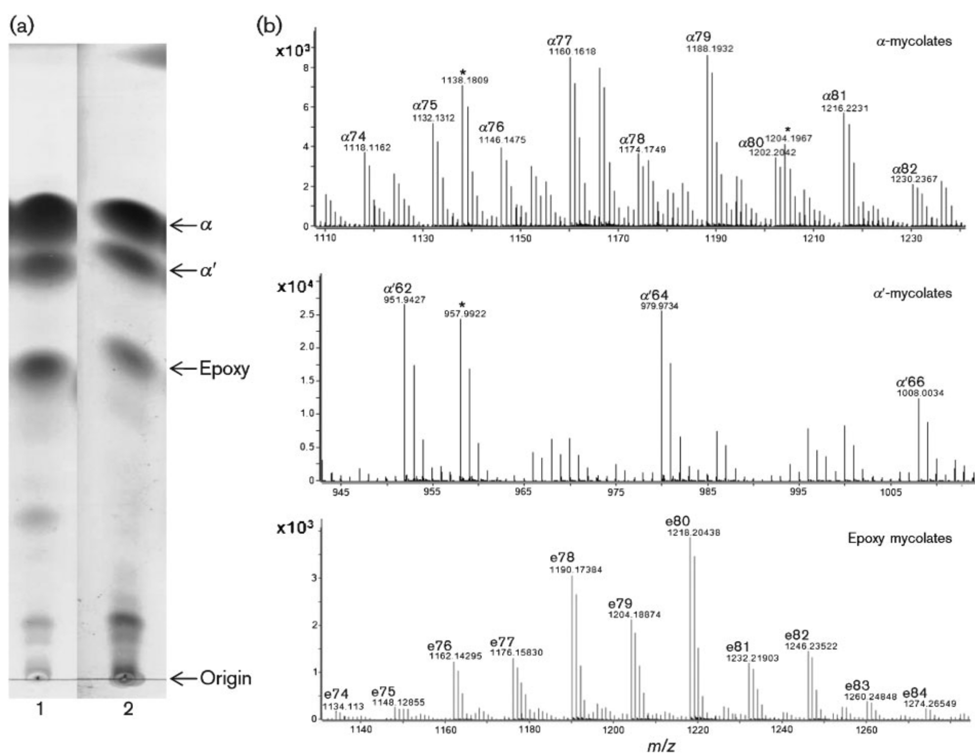

Figure 3. Analysis of MAMEs by TLC and MS. **(a)** TLC fractionation of MAMEs into subclasses: thin-layer chromatogram of MAMEs from whole *M. smegmatis* (lane 1) and Msmeg_1 (lane 2). The development solvent was n-hexane/ethyl acetate (95:5, v/v), used three times. α , α -mycolates; α‘, α’-mycolates; e, epoxy mycolates. **(b)** LC-MS analyses of MAME subclasses extracted from Msmeg_1, carried out in positive ion mode. Top, α-mycolates; middle, α’-mycolates; bottom, epoxy mycolates. The presence or absence of peaks marked with an asterisk (*) was variable and, based on their masses, was probably due to oxidation reactions occurring on the silica gel.

TDM is composed of mycolic acids and a trehalose disaccharide; to confirm the presence of the sugar molecule, we performed alkaline hydrolysis on Msmeg_1. The sugar was extracted and acetylated, and the resulting products were analysed by gas chromatography-MS. In parallel, two standards (MTb TDM and trehalose) were treated exactly as Msmeg_1 sample. Our results showed that the retention time and mass spectra (Fig. 4) obtained for Msmeg_1 and for the MTb TDM standard were the same as the trehalose standard, clearly demonstrating that the sugar in Msmeg_1 is the trehalose. Taken as a whole, the results clearly show that Msmeg_1 is in fact *M. smegmatis* TDM.

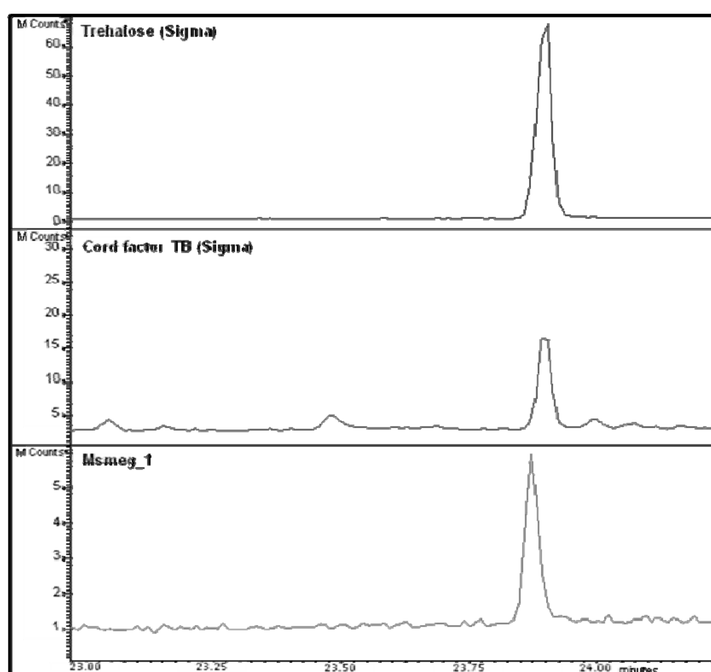

Figure 4. Identification of trehalose in Msmeg_1 and MTb TDM standard by GC-MS..

Treatment of purified Msmeg_1 with LysB

When we analysed pure Msmeg_1, treated and non-treated with LysB, in the positive ion mode by LC-MS, the M + Na pseudo-molecular ions in the region from *m/z* 2160 to 2700 showed the presence of several peak clusters for the non-treated Msmeg_1, while no such ions were observed for the treated sample (Fig. 5). This result is in accordance with our previous TLC

results, in which LysB was able to hydrolyse Msmeg_1. LC-MS analysis (negative mode) for free mycolate in the same samples showed the presence of the expected mycolates in the LysB-treated sample but not in the untreated sample (Fig. 6). These analyses directly showed the action of LysB on Msmeg_1. The mass spectrum of the intact Msmeg_1 (Fig. 5) shows abundant M + Na ions in three clusters with the dominant *m/z* values of 2187, 2395 and 2603 (fully ^{12}C isotope mass). These correspond to the combinations of α'/α', α'/α, and a mixture of α/α and α/epoxy mycolates esterified to trehalose. In Table 1, the most probable combination of mycolic acids present in each molecular species was determined based on the weights of mycolyl residues known to be present from our mycolate analysis (Figs. 3 and 6). Similar heterogeneity in intact TDM molecules from other mycobacterial species has been found previously (Fujita *et al.*, 2005).

Thus, TDM from *M. smegmatis* has now been shown to be a substrate for LysB. In addition, structural details beyond the early initial characterization of TDM in *M. smegmatis* (Mompon *et al.*, 1978) have been obtained. These include the composition of the mycolic acid components (especially the epoxy mycolates) and the determination of which pairs of mycolates are combined on the trehalose disaccharide (Table 1).

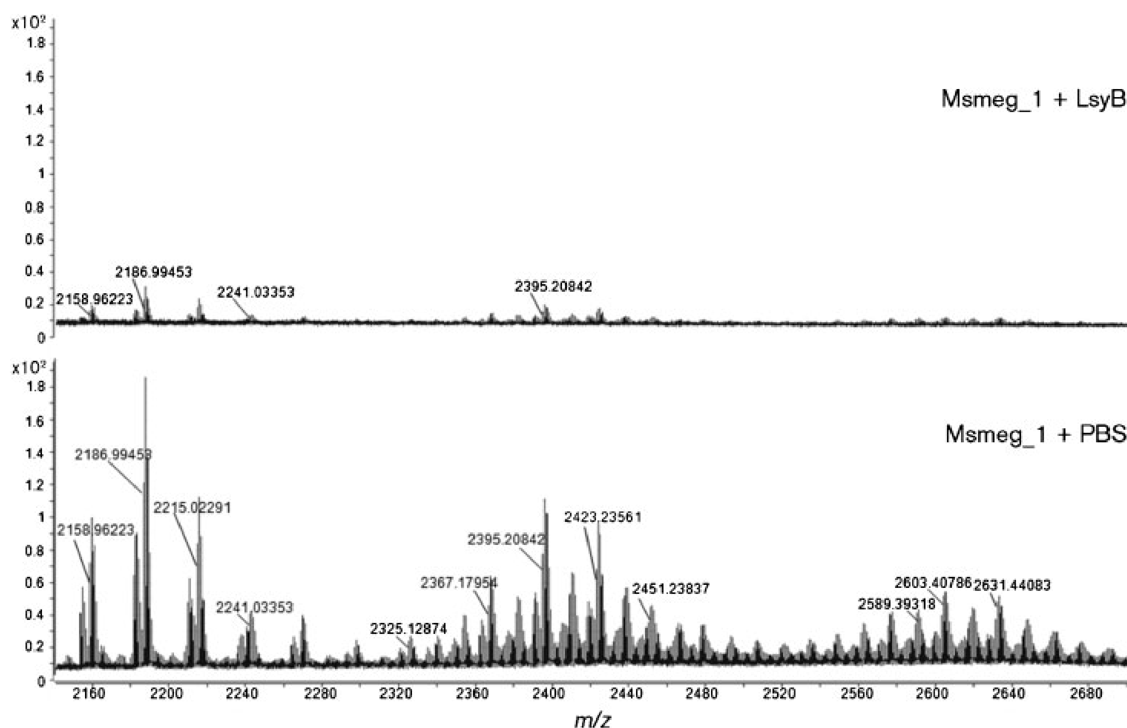

Figure 5. LC-MS analysis (positive ion mode) of purified Msmeg_1 treated with LysB and non-LysB-treated enzyme-treated Msmeg_1. The non-treated Msmeg_1 spectrum revealed the presence of several ion clusters between m/z 2158 and 2647.

Treatment of intact *M. smegmatis* with LysB

Enzymes that attack the mycobacterial cell envelope from the outside are needed as a novel therapeutic method to kill mycobacteria. Such enzymes can also be used as reagents to gain access to the contents of the mycobacterial cell for DNA and RNA analysis, and also for immunohistochemical analysis. Currently, lysozyme has some efficacy for the non-therapeutic purposes but is not as efficient as would be desired and must be used at very high concentrations. Its lack of robust activity makes it an impractical reagent to develop on its own for therapeutic purposes. Although the relatively high molecular mass of LysB (predicted molecular mass of 38.3 kDa) raised the concern that it would not be able to gain access to the mycolate bonds, we did test its ability to inhibit the growth of *M. smegmatis*.

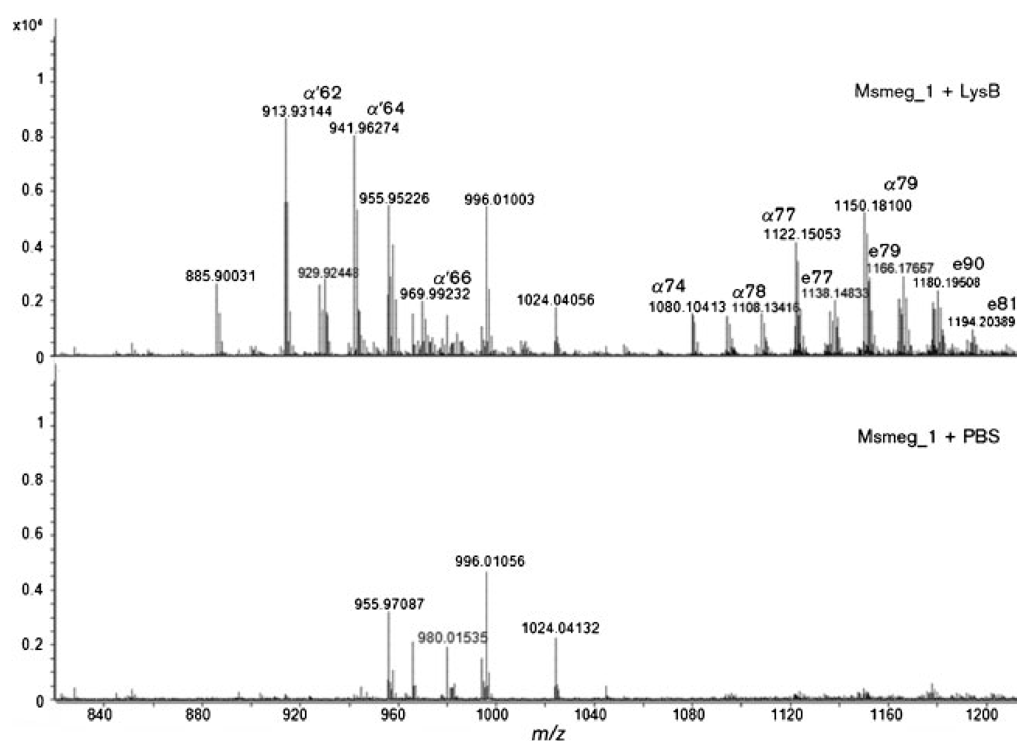

Fig. 6. LC-MS (negative mode) identification of mycolic acids from released Msmeg_1 after LysB treatment (top panel). The bottom panel is a control with no LysB treatment. The mycolates, thus identified, were consistent with the more detailed analysis of TDM shown in Fig. 3.α, α-mycolates; α', α'-mycolates; e, epoxy mycolates. The ion at *m/z* 980 is from the calibration standard; the ions at *m/z* 955, 996 and 1024 (found in both the control and the sample) are of unknown origin, and can also be seen in Fig. 1.

We found that, in the presence, but not in the absence, of Tween 80, LysB effectively killed *M. smegmatis*. Further investigation revealed that this was probably not due to the detergent itself but due to the toxicity of oleic acid released from the Tween 80 by LysB (F. Gil, unpublished observation). Therefore, although we have demonstrated that LysB is able to hydrolyse the

mycolate–arabinogalactan bond and the mycolate–trehalose bond when Ms6 LysB is added from the outside, it is unable to disrupt the outer membrane sufficiently to kill the mycobacteria.

Conclusions

In the present work, we demonstrate that the mycobacteriophage Ms6 LysB lipolytic enzyme is able to hydrolyse ester bonds that are necessary for the integrity of the mycobacterial envelope. In particular, the arabinosyl head groups of the inner leaflet of the outer membrane (ultimately attaching the inner leaflet to peptidoglycan) are cleaved from the mycolates. Importantly, TDM is also de-esterified by the enzyme and, although the exact location of TDM in the cell envelope in not precisely defined, it is likely to be present in the outer leaflet of the outer membrane. It should be noted that the gene encoding LysB is localized in the lytic cassette of Ms6, downstream of the endolysin gene, supporting a role in mycobacterial lysis (Garcia *et al.*, 2002). Thus, we propose that LysB activity, together with LysA acting on the peptidoglycan (unpublished data), results in a more efficient breakage of the cell wall, allowing a robust dissemination of the new phage particles. This is in agreement with a report (Payne *et al.*, 2009) in which a deletion of *lys*B in the mycobacteriophage Giles genome results in a reduction of the mycobacteriophage plaque size, indicating that Giles LysB, although not essential for plaque formation, is required for efficient phage release. Unfortunately, LysB is unable to hinder the growth of *M. smegmatis* when added to growing cultures.

Table 1. The most probable combination of mycolic acids present in TDM of different molecular masses isolated from *M. smegmatis*

Mass no. of TDM (M+Na)*	*M. smegmatis* mycolic acid combination†
2158.96 (C124 Δ2)	α′62 : α′62
2186.99 (C126 Δ2)	α′62 : α′64
2215.02 (C128 Δ2)	α′62 : α′66; α′64 : α′64
2325.12 (C136 Δ3)	α′62 : α74
2353.15 (C138 Δ3)	α′62 : α76; α′64 : α74
2367.17 (C139 Δ3)	α′62 : α77; α′64 : α75
2395.20 (C141 Δ3)	α′62 : α79; α′64 : α77; α′66 : α75
2423.23 (C143 Δ3)	α′62 : α81; α′64 : α79; α′66 : α77
2575.37 (C154 Δ4)	α74 : α80; α75 : α79; α76 : α78; α77 : α77
2589.39 (C155 Δ4)	α74 : α81; α75 : α80; α76 : α79; α77 : α78
2603.40 (C156 Δ4)	α74 : α82; α75 : α81; α76 : α80; α77 : α79; α78 : α78
2617.41 (C157 Δ4)	α75 : α82; α76 : α81; α77 : α80; α78 : α79
2631.44 (C158 Δ4)	α76 : α82; α77 : α81; α78 : α80; α79 : α79
2661.43 (C159 epoxy Δ3)	α82 : e77; α81 : e78; α80 : e79; α79 : e80; α78 : e81; α77 : e82; α76 : e83; α75 : e84
2675.43 (C160 epoxy Δ3)	α82 : e78; α81 : e79; α80 : e80; α79 : e81; α78 : e82; α76 : e84; α77 : e83; α76 : e84

*The C number refers to the number of carbons in the mycolate chains (does not include the 12 carbons of trehalose). The D number indicates the total number of double bonds. †, α-mycolates; α', α'-mycolates; e, epoxy mycolates.

Of note is the fact that LysB hydrolyses the *M. smegmatis* TDM, a glycolipid present in the lipid outer layer of mycobacteria, which has been associated with virulence in pathogenic bacteria. Our results suggest that TDM from the slow-growing mycobacteria are also hydrolysed by LysB, indicating that homologous proteins from other mycobacteriophages will also hydrolyse this glycolipid. It has already been shown that mycobacteria can survive after removal of the TDM, but viability of these bacteria inside macrophages is significantly reduced (Indrigo *et al.*, 2002).

4

The role of LysB in Ms6 infection cycle

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Manuscript in preparation

Summary

All dsDNA phages encode two proteins involved in host lysis, an endolysin and a holin. In addition, some bacteriophages encode additional proteins like the Rz and Rz1 present only in bacteriophages that infect Gram-negative bacteria. Recently it was identified in mycobacteriophages a gene located in the lytic cassette, encoding a protein with mycolyl arabinogalactan esterase activity. Taking in consideration the bacterial complex cell wall that mycobacteriophages encounter during their life cycle it is interesting to evaluate the role of these proteins in lysis. In the present work we used a novel recombineering technique, BRED, that allowed us to engineer an Ms6 mycobacteriophage defective on *lysB* gene and an Ms6 mycobacteriophage producing a LysB protein with a His_6-tag. It was determined that LysB production begins 120 minutes post infection. $Ms6_{\Delta lysB}$ was able to form plaques indicating that *lysB* gene is not essential for Ms6 viability. Comparison between $Ms6_{\Delta lysB}$ and $Ms6_{Wt}$ showed no differences in the plaque size with both phages having a similar lysis kinetic. Moreover, the burst sizes are also similar. Ms6 *lysB* defective showed that although it is not observed a delay of lysis when compared with the Ms6 wild type, it takes more time to accomplish a full host lysis.

Introduction

Double stranded DNA phages must lyse their hosts in order to guarantee their own survival in the biosphere. The main barrier to the release of the new phage particles at the end of a lytic cycle is the bacterial cell wall and thus, compromising this barrier is the main goal for the lytic process. To achieve lysis, phages need to synthesize at least two essential proteins: an endolysin, a protein designed to attack at least one of the three types of the covalent bonds in the peptidoglycan; and a holin, a small protein that accumulate in the cytoplasmic membrane and a have a role in the timing of lysis (Young, 1992; Young *et al.*, 2000; Gründling *et al.*, 2001). Some holins form pores large enough to allow the passage of endolysins that have accumulated in the cytoplasm. This is the case of one of the most well studied bacteriophage, the phage λ (Young, 1992). In other cases, endolysins are endowed with signal sequences that, with the participation of the host sec-system are translocated to the cytoplasmic membrane to be positioned in the periplasm. Examples are the endolysins of phage phages P1 and the lambdoide phage 21, which carry an N-terminal signal arrest release (Wang *et al.*, 2000; Young *et al.*, 2000; Xu *et al.*, 2004; Xu *et al.*, 2005) and Lys44, the endolysin of the *Oenococcus* phage fOg44, which carries a typical cleavable signal peptide (São José *et al.*, 2000). In these examples, although the holin is not required for endolysin export, its role in lysis is still essential to define the timing of lysis.

In addition to these essential genes, most phages infecting Gram-negative bacteria contain additional genes whose products are involved in disrupting the outer membrane. One of the most well studied are the λ *Rz* and *Rz1* genes (Young, 2006). *Rz* encodes a class II inter membrane protein with a periplasmic C-terminus. The *Rz1* coding region is embedded out of frame within the *Rz* gene and encodes an outer membrane lipoprotein with the lipolyl moieties inserted in the inner leaflet of the outer membrane. It was proposed that these two proteins interact by virtue of their C-terminus, forming a complex that spans the entire periplasm, providing a physical link between the inner and outer membrane. This would cause the cytoplasm and the environment to become topologically equivalent and, thus all conceivable barriers to the release of the progeny virions would be eliminated (Berry *et al.,* 2008). In addition to λ, Rz/Rz1 homologues were also described in other phages infecting Gram-negative bacteria (Summer *et al.,* 2007). In the study performed by Summer *et al.* (2007), functional homologous of Rz/Rz1 encoded by a single gene were also identified in some phages; these new class of proteins, named spanins, are functionally equivalent to the Rz/Rz1 proteins.

Taking in to consideration that Gram-negative bacteria possess an external membrane, one can understand why bacteriophages that infect these bacteria would have the necessity to encode additional genes with the purpose of achieving a more efficient lysis. The process of host lysis in λ was suggested to comprise three steps that compromise the inner membrane (IN), the peptidoglycan (PG) layer, and the outer membrane (OM), reflecting the order of genes involved (Berry *et al.*, 2008). The first step would comprehend the holin harmless accumulation in the IM followed by sudden formation of extremely large and irregular holes. Besides terminating with macromolecular synthesis and virion assembly, this step would allow the phage endolysin to gain access to the peptidoglycan and its degradation, accomplishing step two. PG destruction is followed by a third step that involves disruption of the outer membrane, which requires Rz and Rz1 action. The same thoughts could cross our minds when we think in the lytic cassettes of phages that infect mycobacteria that also possess an outer membrane, with LysB-like proteins might having a similar role to Rz and Rz1 proteins. Although mycobacteria are classified as Gram-positive bacteria they are unusual in possessing a mycolic acid-rich outer membrane that is covalently attached to an arabinogalactan-peptidoglycan complex (Daffé, 2008). Our group has been focused in studying the lysis mechanism of mycobacteriophage Ms6, a *Siphoviridae* phage that infects *Mycobacterium smegmatis*. The Ms6 lytic cassette encodes five proteins (Garcia *et al.,* 2002). In addition to the holin and the endolysin, Ms6 codes for: (i) a chaperone (Gp1) that was demonstrated to interact with the N-terminal sequence (60 amino acids) of the Ms6 endolysin, its effector (Catalão *et al.*, 2010); (ii) an additional lysis protein, LysB, recently identified as a lipolytic protein, that specifically targets the lipid layer of the mycobacterium complex cell wall (Gil *et al.,* 2008; Gil *et al.,* 2010), cleaving the mycolyl arabinogalactan bond to release free mycolic acids (Payne *et al.,* 2009, Gil *et al.,* 2010); (iii) and another protein with some holin features (Gp5) which was suggested that combined with Gp4 would play the role of a holin, since expression of both proteins is necessary to effect lyse the host at the correct and programmed timing (Catalão *et al.,* 2011a).

LysB homologous proteins were identified in mycobacteriophages genomes as well as in phages that infect *Rhodococcus equi* (Summer *et al.,* 2011). From the 81 compared mycobacterial genome sequences, 77 encode a LysB-like protein (Hatfull *et al.,* 2010, Henry *et al.,* 2010).

In the present work we investigated the LysB role on the Ms6 lytic cycle through the analysis of the biological parameters of a *Mycobacterium smegmatis* infection with an Ms6 derivative mutant deleted in *lysB* gene.

Materials and Methods

Bacterial strains, phages, plasmids and culture conditions

Bacterial strains, phages, plasmids and oligonucleotides used throughout this study are listed in Table 1. *M. smegmatis* strains were grown at 37 °C in 7H9 medium (BDBiosciences) with shaking or Middlebrook 7H10 (BDBiosciences), supplemented with glucose and $CaCl_2$.

Table 1. Strains, bacteriophages and plasmids used in this study

Strains, plasmids, bacteriophages or oligonucleotides	Description	Source or reference
Bacteria		
Mycobacterium smegmatis mc²155	High-transformation-efficiency mutant of *M.smegmatis* ATCC 607	Snapper *et al.* (1990)
Bacteriophages		
$Ms6_{Wt}$	Temperate bacteriophage from *M. smegmatis*	Portugal *et al.* (1989)
$Ms6_{\Delta lysB}$	996 bp in-frame deletion of the Ms6 *lysB* gene	This study
$Ms6_{lysB\mathrm{His6Tag}}$	His_6Tag insertion at the 3' end of the Ms6 *lysB* gene	This study
Plasmid		
pJV53	Derivative of pLAM12 with Che9c *60* and *61* under control of the acetamidase promoter; Kan^r	Van Kessel, J. C. & Hatfull, G. (2007)
Oligonucleotides	**Sequence 5'-3'**	
PrΔ*lysB*	CTCGGCGGAAAAACCCTCCTCGTGGACGCG GTAGCAGAACTGTTGGGCCACTGATAGGAG GCACCCATGCTGACACGTTCATTCTGGATCG ACGCCGCCGAGCG	Ms6 *lysB* 996 bpΔ
PrExtΔ*lysB*Fw	CGAGATCCTGCGGCAACTGCGCGGATACAA CCTCACTGGCTGGCCGCAGCTCGGCGGAAA AACCCTCGTGGACG	Extend PrΔ*lysB*
PrExtΔ*lysB*Rv	CCCCGGCGCCGAGGGTGGCGATCGCGGTTT GGGCGAATGTGCGTATGGCACGCTCGGCGG CGTCGATCCAGAATG	Extend PrΔ*lysB*

DADA Δ*lysB*-PCR	GCGCTAGCAGAACTGTTGGGCCACTGATAG	Ms6Δ*lysB*
DADA Ms6-PCR	CGTCTCGTACTGCACGTACCGGTTCTTC	Ms6Δ*lysB*; Ms6*lysB*His6
Pr*lysB*His_6Tag	CTACAACCCCCAGCCCGCCATCGACTACCT ACGCACACACCACCACCACCACCACTGAG AGGCACCCATGCTGACACGTTCATTCTGG ATCG	Ms6 *lysB*His_6Tag insertion
PrExt*lysB*His_6Fw	CTCGACGCCGGCATGTTCTTCGCGAAACGC ACCGGCCCGCACGTGGACTACAACCCCCAG CCCGCCATCGACTAC	ExtendPr*lysB*His_6Tag
PrExt*lysB*His_6Rv	GTGGCGATCGCGGTTTGGGCGAATGTGCGT ATGGCACGCTCGGCGGCGTCGATCCAGAAT GAACGTGTCAGCAT	ExtendPr*lysB*His_6Tag
DADA *lysB*His_6-PCR	CCTACGCACACACCACCACCACCAC	Ms6 *lysB*His_6Tag

Ms6 bacteriophage amplification and harvesting

A standard plaque assay was performed and the plates were used to harvest phage. High-titre phage suspension was obtained by adding 5 ml of SM buffer (10 mM Tris-HCl; pH 7.5; 10 mM $MgSO_4$; 68.5 mM NaCl; 1 mM $CaCl_2$) to a plate from a plaque assay for an overnight incubation at 4 °C. The buffer was then removed, centrifuged (1000 *g* for 10 min) and the supernatant was filtered through a 0.2-µm filter. The approximate phage titre of the suspension was subsequently evaluated using a serial dilution method.

Construction of Ms6 mutant phages

Construction of Ms6 mutant phages was performed using Bacteriophage Recombineering of Electroporated DNA (BRED) in *M. smegmatis*. Recombineering substrates and BRED strategy were done as described previously (Marinelli *et al.,* 2008; Catalão *et al.,* 2010). Briefly, for deletion of the Ms6 *lysB,* a 100 bp oligonucleotide, PrΔ*lysB*, that has 50 bp of homology upstream and downstream of the region to be deleted was extended by PCR using two 75 bp extender primers, PrExtΔ*lysB*Fw/ PrExtΔ*lysB*Rv, which have 25 bp of homology to the ends of the 100-mer and add an additional 50 bp of homology on either end. For the His_6-tag insertion a 92 bp oligonucleotide (Pr*lysB*His_6tag) was extended with primers PrExt*lysB*His_6Fw/

PrExt*lys*BHis$_6$Rv. The final 200 bp dsDNA products were purified using MinElute PCR Purification Kit (QIAGEN) and co-electroporated with Ms6$_{Wt}$ DNA into electrocompetent recombineering cells of *M. smegmatis* mc^{2}155:pJV53. Cells were resuspended in 7H9 supplemented with 0.5% glucose and 1 mM of $CaCl_2$, incubated at 37 °C for 2 h (prior to lysis) and plated as top agar lawns with *M. smegmatis* mc^{2}155. Phage plaques were picked into 100 µl SM buffer, eluted for two hours at room temperature and analysed by Deletion Amplification Detection Assay (DADA)-PCR (Marinelli *et al.,* 2008) with primers DADA Δ*lysB*-PCR/ DADA Ms6-PCR to detect *lysB* deletion or with primers DADA *lysB*His$_6$-PCR/ DADA Ms6-PCR. Mixed primary plaques containing both the mutant (either *lysB* deletion or His$_6$Tag insertion) and the wild type DNA were eluted as described above, and serial dilutions were plated with *M. smegmatis.* Individual secondary plaques or lysates were screened by DADA-PCR for the presence of pure *lysB* deletion or *lysB* His$_6$ Tag insertion mutant phages.

LysB expression in M. smegmatis-infected cells

Examination of LysB synthesis in *M. smegmatis* was performed as previously described (São-José *et al.,* 2000; Catalão *et al.*, 2010). An exponential growing culture of *M. smegmatis* mc^{2}155 was infected with Ms6$_{LysBHis6}$ at an approximate multiplicity of infection (m.o.i) of 10 and incubated for 30 minutes at 37 °C for phage adsorption. 10 ml samples were withdrawn at 30 min intervals for 240 min, cells were pellet by centrifugation and frozen at -20 °C. After thawing, cells were concentrated 100-fold in phosphate buffer saline (PBS) supplemented with 20 mg of lysozyme per ml. After an incubation period at 37 °C for 1 h, 25 µl of 5× SDS-PAGE sample buffer were added followed by incubation at 100 °C for 5 min to complete cell lysis. *M. smegmatis* extracts were analysed by western-blotting and LysB immunodetection was performed using horseradish-peroxidase-conjugated anti-His monoclonal antibody (Roche).

Lysis assay and burst size determination

The lysis assay was performed as described in Payne *et al*. (2009) with some modifications. Briefly, *M. smegmatis* cells were grown in 7H9 supplemented with glucose and 1mM $CaCl_2$ to an OD_{600} 0.6-0.8. Cells were infected with phage particles at an m.o.i. of 100 and incubated for 30 min at 37 ºC. After phage adsorption, an aliquot of 100µl was collected (T0), and incubation continued at 37 ºC with shaking. 150µl alliquots were collected every 30 min for 5 h. ATP release was measured by addition of 50 µL of ENLITEN rLuciferase/Luciferin reagent (Promega), and luminescence recorded in a Monolight 2010 luminometer Tecan.

The burst-size determination was adapted from protocols described by Adams (1959). Briefly, *M. smegmatis* cells were used in exponential growth using an m.o.i. of 1. *M. smegmatis* cells were pelleted and resuspended in 1 ml of a phage suspension ($Ms6_{Wt}$ or $Ms6_{\Delta LysB}$) supplemented with 1 mM $CaCl_2$. The mixture was incubated 50 min at 37 °C to allow adsorption of the phages. 100 µl of 0.4% H_2SO_4 was added to inactivate the non-adsorbed phages and the incubation continued for 5 min. The suspension was neutralized with 100 µl of 0.4% NaOH and 10 µl of infected cells were diluted in order to obtain ≤ one infected cells ml^{-1} in 7H9 supplemented with 0.5% glucose and 1 mM $CaCl_2$. Samples of 1 ml of infected culture were distributed in 50 tubes and incubated for 4 h at 37 °C. 200 µl of *M. smegmatis* cells and top agar (4 ml) were added to each tube and plated on 7H10. After 24 h at 37 °C, the phage plaques were counted and the distribution of *Poisson* [P(*n*)] was applied to determine the burst-size (BS): P (n) = ($e^{-c} . c^n$)/ n! (e < 1), where P (n) is the probability of samples having *n* infected cells and ***c*** is the average number of infected cells per tube; and BS = (total plaque count in the 50 plates) / (total number of infected cells).

Results and Discussion

Ms6 LysB is not essential for phage viability

To better understand the contribution of LysB to the Ms6 infection cycle, we first asked whether it is an essential function for plaque formation. We used the Bacteriophage Recombineering of Electroporated DNA (BRED) strategy to delete *lysB* gene from the mycobacteriophage Ms6 genome (Marinelli *et al.,* 2008). Using this technique we were able to obtain mutant phages demonstrating that $Ms6_{\Delta lysB}$ is viable (recombineering efficiency of 28%) (Fig.1) and that LysB is not required for plaque formation (Fig. 2).

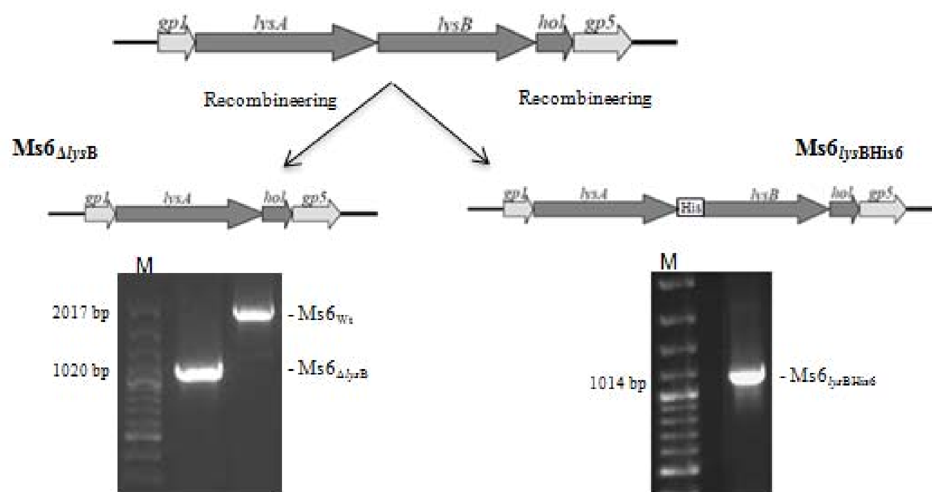

Figure 1. Strategy for construction of Ms6 *lysB* mutants. The primers used to detected the $Ms6_{lysBHis6}$ were specific for the hexahistidine region, that is why the Ms6 wild type was not amplified.

Moreover, no significant differences were observed between plaques produced by $Ms6_{wt}$ and by $Ms6_{\Delta lysB}$ (Fig. 2).

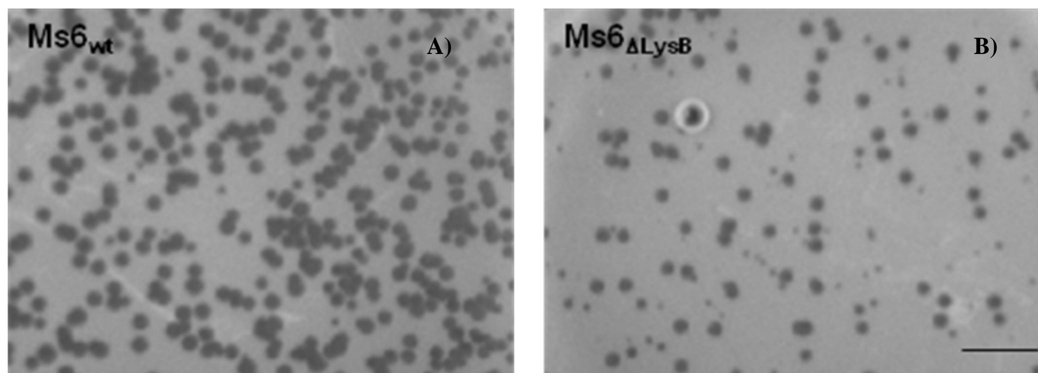

Figure 2 Plating ability of the *lys*B mutant bacteriophages. A) *M. smegmatis* infected with $Ms6_{wt}$ phage; B) *M. smegmatis* infected with $Ms6_{\Delta LysB}$. Scale bar indicates 1 cm.

This result is not in agreement with the one obtained for the only other LysB characterized so far (Payne, *et. al* 2009). As it happens with $Ms6_{\Delta LysB}$, deletion of *lys*B gene in mycobacteriophage Giles does not affect phage viability or plaque formation, however in contrast with these authors we did not observe a reduction in the plaque size.

Effect of Ms6 LysB on phage lysis

In order to compare the progression of phage infections of $Ms6_{Wt}$ and $Ms6_{\Delta lysB}$ we measure ATP release which may reflect either lysis or permeabilization of the cell wall. Infection of *M.*

smegmatis with $Ms6_{Wt}$ and $Ms6_{\Delta lysB}$ results in ATP release after 2h30 min, 2 h represented in the graphic plus 30 min of adsorption (see methods for detail). Even though we cannot observe any delay on the beginning of lysis, $Ms6_{\Delta lysB}$ does not show a similar progression of infection as the $Ms6_{wt}$ (Fig. 3). Our results are not totally in agreement with the ones obtained for mycobacteriophage $Giles_{\Delta lysB}$ since deletion of *lys*B is responsible for a 30 min delay in cell lysis (Payne *et al.*, 2009).

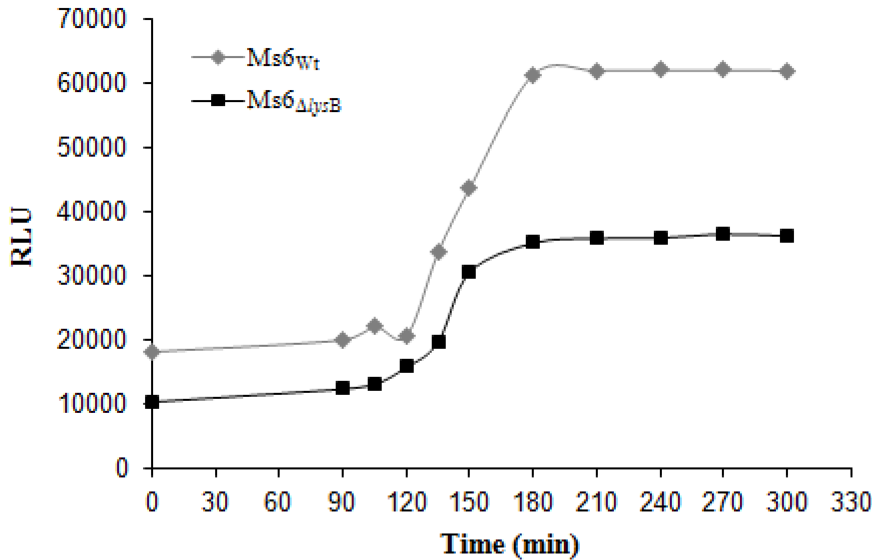

Figure 3. *M. smegmatis* mc²155 cells were infected with $Ms6_{Wt}$ and $Ms6_{\Delta lysB}$ and ATP release measured at different times after infection. ATP was measured in a luciferase assay and reported as relative light units (RLU).

Of note is the fact that Ms6 and Giles have different lytic cassettes (Fig. 4) (Morris *et al.*, 2008; Hatfull *et al.*, 2010). The identified lysis genes in Giles genome are *gp31* encoding LysA and *gp32* encoding LysB but no holin or other lysis functions were so far assigned to any other Giles gene (Morris *et al.*, 2008; Hatfull *et al.*, 2010). In addition to the LysA, LysB and holin Ms6 encodes Gp1, a chaperone-like protein that transports the endolysin across the cytoplasmatic membrane in a holin-independent manner and is required to accomplish an efficient lysis of *M. smegmatis* (Catalão *et al.,* 2010); and Gp5, a protein with holin-like function (Catalão *et al.,* 2011a). At the present moment we do not know how these different cassettes contribute to the different phenotypes observed with $Ms6_{\Delta lysB}$ and $Giles_{\Delta lysB}$.

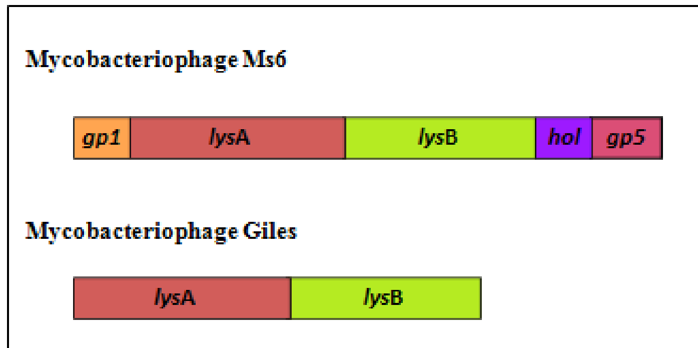

Figure 4. Schematic representation of mycobacteriophage Ms6 and Giles lytic cassette.

Single-burst experiments were done to compare the viable progeny released from single cells infected with $Ms6_{Wt}$ and the $Ms6_{\Delta lysB}$ phages. In our experimental conditions, $Ms6_{\Delta lysB}$ yielded a similar burst size to what was described for $Ms6_{Wt}$, an average of 149 viable phages from one bacterium (Catalão *et al.*, 2010). This result is in agreement to what is observed in the plaque size. However, the minor ATP release observed with $Ms6_{\Delta lysB}$ does not reflect a minor phage number release. At this point it is not clear how a deletion in Ms6 *lysB* gene affects the phage growth parameters; however it is noteworthy that our attempts to obtain a one-step growth curve were unsuccessful since we observed great variation between the results. How this reflects a defect in the phage release remains to be determined.

LysB synthesis in the course of *M. smegmatis* infection

To analyse the LysB production in *M. smegmatis,* the BRED strategy was also used to obtain an Ms6 phage with the 3' end of *lys*B fused to a sequence coding for a hexahistidine tag, allowing the production of a LysB-His_6 tagged protein. (Fig. 1). After *M. smegmatis* infection with $Ms6_{lysBHis6}$, extracts were prepared from samples taken at 30 min intervals as described previously in materials and methods. The samples were analysed by western-blotting and LysB synthesis was detected with an antibody against the His_6-tag. As observed in Fig. 5 LysB synthesis could be detected beginning at 120 min postinfection. As expected, this result is similar to the one obtained for LysA synthesis (Catalão *et al.*, 2011b), since both proteins are transcribed into the same mRNA (Garcia *et al.,* 2002).

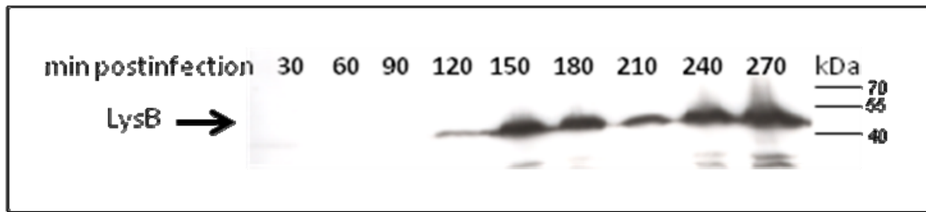

Figure 5. Time course of LysB synthesis during Ms6$_{lysB\text{His6}}$ infection of *M. smegmatis*. The molecular weight in kDa of LysB is indicated on the left; position of LysB protein is indicated by an arrow on the right. Only the results for 30 to 270 min postinfection are shown.

The fact that genes for Rz/Rz1 equivalents are found in nearly all phages of Gram-negative hosts (Summer *et al.,* 2007) indicates that for the conditions found in nature, the outer membrane is a significant barrier even after holin-endolysin mediated destruction of the cell wall. A similar point of view could be proposed for *lysB*-like genes that are found in nearly all mycobacteriophages, present in 77 out of the 81 described mycobacteriophages genomes (including mycobacteriophage Ms6) (Hatfull *et al.,* 2010; Henry *et al.,* 2010), suggesting that mycolylarabinogalactan layer might be a significant barrier even after the endolysin mediated destruction of the cell wall. It was suggested that the 4 mycobacteriophages that do not encode a LysB-like protein, would have evolved to utilize a host-encoded cutinase-like enzyme for this function (Hatfull, 2010). Although we cannot observe a delay on the lysis timing with the Ms6$_{\Delta lysB}$, this phage has a slower lysis rate. Taking in consideration that LysB is a lipolytic enzyme with mycolylarabinogalactan esterase activity, we cannot rule out that in nature, in different conditions than the ones performed in the laboratory, the absence of this protein would have a more accentuated impact in the Ms6 lysis profile. Of note, is the fact that for many years the precise role of the λ Rz/Rz1 proteins was not fully known, since under usual laboratory conditions, Rz/Rz1 are not required for bacteriophage λ lysis (Young & Wang, 2006). Recently, LysB homologues were identified in *Rhodococcus equi* phages (Summer *et al.*, 2011). Considering that *Rhodococcus* and *Mycobacterium* share the same complex envelope, an outer layer composed of mycolic acids covalently linked to the peptidoglycan (Sutcliffe, 1997), it is anticipated that these phages should exhibit some of the features characteristic of mycobacteriophages, such as LysB that attacks this outer layer. However, the genetic structure of the lysis genes of the *Rhodococcus* phages differed significantly from that of the mycobacteriophage in that they lacked well-defined lysis gene cassettes.

Our present knowledge of the mechanism of bacteriophage lysis suggests that the complexity of phage lytic cassettes depends on their hosts. Hosts with a simpler envelope require the phage to possess a simple lytic cassette, whereas hosts with a complex envelope require the phage to have a more complex lytic cassette.

5

CONCLUDING REMARKS

The results presented in this thesis allowed the characterization of the mycobacteriophage Ms6 LysB protein and its role in *Mycobacterium smegmatis* lysis and allowed the following conclusions:

- LysB is a lipolytic protein encoded by a gene located within the lytic cassette of a mycobacteriophage. This protein is able to hydrolyze both short and long-chain substrates, showing preference to long-chain substrates. LysB could not be classified as lipase or esterase, suggesting that LysB and homologous proteins could form a new family of lipolytic enzymes.
- The LysB main target on the mycobacteria cell envelope is the mycolyl-arabinogalactan complex. LysB was characterized as a mycolyl arabinogalactan esterase since it was able to hydrolyze the bond between the mycolic acids and the arabinogalactan. In addition, LysB activity is not specific, since it can hydrolyze the TDM of *M. smegmatis*, *M. tuberculosis* H37Ra and *M. bovis* BCG
- Even though an Ms6 mycobacteriophage defected of *lysB* gene was viable and the beginning of lysis occurs at the same time as the $Ms6_{Wt}$, $Ms6_{\Delta lysB}$ had a slower progression to accomplish total lysis.

This work represents a step forward in understanding the mechanisms involved in the mycobacteriophage lysis. It was described, for the first time, a gene within the lytic cassette of the mycobacteriophage Ms6, which encodes an enzyme with lipolytic activity. The starting point for this work was the presence of a conserved pentapeptide motif characteristic of lipolytic enzymes which lead us to search for lipolytic activity. Since lipolytic enzymes include carboxylesterases and true lipases (Arpigny & Jaeger, 1999), activity of the recombinant protein His_6-LysB was tested on plate assays with esterase and lipase specific substrates. LysB was able to hydrolyze tributyrin, Tween 20 and Tween 80 (esterase substrates) as well triolein and rhodamine B (true lipase substrates). Another evidence of true lipase activity was obtained with the release of resorufin from the triglyceride 1,2-*O*-dilauryl-*rac*-glycero-3-glutaric acid resorufin ester. More specific assays with a wide range of p-nitrophenyl fatty acid esters were performed and LysB showed activity with all substrates tested, demonstrating a higher affinity for pNP esters of longer chain length (C_{16} and C_{18}). These results, together with the higher catalytic efficiency revealed for C_{12} and C_{14} pNP esters, indicate that the long chain substrates must represent the natural substrate of LysB, suggesting that this enzyme could be classified as a lipase. However, the amino acid sequence of LysB does not show the characteristic features of any of the lipases families described so far (Arpigny & Jaeger, 1999). LysB conserved

pentatpeptide (Gly-Tyr-Ser-Gln-Gly) resembles the pentapeptide described in most cutinases, nevertheless LysB amino acid sequence lacks the pattern described around the conserved motif (Parker *et al.,* 2007). Several conserved block of amino acids were identified on LysB and mycobacteriophage homologous proteins. These observations together with the fact that it was not possible to group LysB and putative proteins on any other lipolytic enzyme family, lead us to suggest that they might be part of a new family of lipolytic enzymes.

In addition, conserved potential amino acids residues involved in the catalytic triad were also observed in the amino acid sequence alignment between Ms6 LysB and its homologous proteins. Substitutions of Ser168 (located at the center of the pentapeptide GXSXG), Asp215 and His246 by an Alanine resulted in a decrease of the enymatic activity, indicating that those amino acids are involved in catalysis. The involvement of Serine in the LySB activity was further strengthened by the decreased in enzyme activity after treatment with PMSF, a serine protease inhibitor. The results demonstrated that Ser168 is part of the catalytic triad, since almost no activity was observed on both assays and that Ms6 LysB belongs to the family of serine hydrolases, as is the case of lipid hydrolases (Holmquist, 2000).

One can question what would be the role of a lipolytic enzyme in cell lysis. Since *lys*B gene was located within the lytic cassette of mycobacteriophage Ms6, and since LysA was already identified as an amidase, together with the fact that 60% of the mycobacteria cell wall is composed of lipids (Brennan & Nikaido, 1995) LysB activity was tested towards the lipid layer of *M. smegmatis*. After treatment of *M. smegmatis* mAGP with LysB it was observed an increase in free mycolic acids by LC-MS. These results demonstrated that LysB was able to hydrolyze ester bonds that are necessary for the integrity of the mycobacterial envelope which is in agreement with the work performed by Payne *et al.,* (2010), where the authors demonstrated that LysB from the mycobaceriophage Giles has mycolyl arabinogalactan esterase activity. Even though these homologous proteins share a low identity within the amino acid sequence (15% of identity), they have in common the conserved motif GXSXG of lipolytic enzymes (Hatfull *et al.,* 2006; Gil *et al.,* 2010), and share the conserved amino acids Asp and His suggesting a common function and thus a common substrate for them. Until very recently, *lysB* homologues were only found within mycobacteriophage lysis cassettes. Summer *et al.,* (2010) identified for the first time LysB-like proteins in phages that infects *Rhodococcus equi*. One of the features that *Rhodococcus* shares with *Mycobacterium* is the presence of an outer layer composed of mycolic acids covalently linked to the peptidoglycan (Sutcliffe, 1998). This layer presents an additional challenge to phages infecting the mycolata group, both in terms of adsorption and DNA injection process as well as host cell lysis. One can hypothesize that to overcome this barrier mycobacteriophages encode LysB equivalents and the same can be anticipated for

phages that infect other members of the *Corynebacterineae* suborder, including *Rhodococcus*, which would also carry genes encoding LysB equivalents (Summer *et al.,* 2010).

In addition to a mycolyl arabinogalactan esterase acitvity, Ms6 LysB also acts on other lipids containing mycolic acids. It was shown that after treatment of *M. smegmatis* total lipids, one other lipid was affected by LysB activity. Structural analysis identified the lipid as the mycobacterial TDM, which is composed by two mycolic acids molecules bound to a trehalose molecule by an ester bond. After LysB activity, free mycolic acids and free trehalose molecules were identified, indicating that LysB hydrolyzed the ester bond of the TDM. Nevertheless, due to the importance of the mAGP-complex on stability of the mycobacteria cell envelope it seams reasonable to considerer that the cleavage of the mycobacterial outer membrane from the peptidoglycan-arabinogalactan layer is the primary role of LysB. In contrast to what happens with most of the described endolysins which, are highly specific towards their hosts (Loessner, 2005), LysB is not specific, since it hydrolyses the TDM from both fast and slow growing mycobacteria, like *M. tuberculosis* and *M. bovis* BCG.

After characterizing LysB as a lipolytic enzyme and demonstrating that its natural substrate is the mycolyl-arabinogalactan complex, the next step was to understand the role of this protein during mycobacteriophage life cycle. An Ms6 derivative mutant, lacking *lysB* gene was shown to be viable, indicating that LysB is not essential for plaque formation. Although phage release can be detected at the same time as the $Ms6_{Wt}$, $Ms6_{\Delta lysB}$ appears to have a slower phage release rate, indicating that accomplish total lysis later than the wild type.

$Ms6_{\Delta lysB}$ can destroy the peptidoglycan because it still produces the endolysin, but without LysB the mash of lipids that are not hydrolysed certainly difficult the phage release, resulting in a delay of phage release. Taking in consideration all the above LysB characteristics, one could have hypothesized that *lysB* deletion would have a greater impact in Ms6 lysis parameters (burst size, plaque size and lysis timing). However, we cannot rule out that in nature, in different conditions than the ones reproduced in a laboratory environment, this protein would have a more significant role during Ms6 infection. Our results are not completely similar to the ones obtained for the only other LysB protein described so far (Payne *et al.*, 2009) but it must be taken in consideration that the lytic cassettes of both mycobacteriophages are different, and so far we do not know how the additional genes can impact on the mycobacteriophages lysis.

Mycobacteria are considered to be Gram-positive bacteria, however the existence of the mycobacterial outer membrane composed of mycolic acids and free lipids presents a barrier

analogous to the outer membrane of Gram-negative bacteria (Hoffmann *et al.*, 2008; Zuber *et al.*, 2008). Analogies can be made between the function of LysB against the mycobacterial outer membrane with the lambda Rz/Rz1 homologues of Gram-negative hosts, which mediate the final step of host lysis by fusing the inner and outer membranes (Berry *et al.,* 2008; Payne *et al.,* 2009; Summer *et al.,* 2007). So, bacteriophages infecting mycobacteria may also need additional lysis genes to accomplish lysis. Due to the complexity of the cell wall of mycobacteria, it is easy to understand why a mycobacteriophage would developed an alternative solution, a lipolytic enzyme, to compromise the mycobacterial outer membrane by compromising its linkage to the arabinogalactan-peptidoglycan layer, in order to meet one the principles of phage lysis mentioned by Ry Young: 'the lysis process, once initiated, should be rapid in order to improve phage production' (Young, 2005).

The sequence analysis of 81 mycobacteriophage genomes (Hatfull *et al.,* 2010; Henry *et al.,* 2010) revealed that 77 mycobacteriophages encode a LysB-like protein, which demonstrates the importance of the protein throughout the mycobacteriophage evolution indicating that the mycolyl arabinogalactan layer is a significant barrier even after the endolysin mediated destruction of the cell wall. Thus, we propose that LysB activity, together with LysA acting on the peptidoglycan results in a more efficient breakage of the cell wall, allowing a robust dissemination of the new phage particles. Of note are the four mycobacteriophages (Che12, Rosebush, Qyrzula and Myrna) that lacks a LysB-like protein. A hypothesis is that these phages have evolved a mechanism for utilizing a host-encoded cutinase-like enzyme for this function (Hatfull, 2010).

At this time it is unknown how Ms6 LysB is localized to its substrate, since no signal sequences allowing its transport across the cell barriers have been identified (Gil *et al.,* 2008). Since Ms6 LysA access to the peptidoglycan is not holin dependent, but in fact is the Ms6 Gp1 chaperone that leads LysA to it substrate, it would be interesting to see if LysB is also dependent on the Gp1 chaperone to access the lipid layer after the peptidoglycan. Recently, our group demonstrated the LysA antibacterial activity when added to several mycobacteria species (Catalão *et al.*, 2011a). It would be interesting to see if the combination of LysA and LysB would have an increased effect since when Ms6 LysB alone is added from the outside, it is unable to disrupt the outer membrane sufficiently to kill the mycobacteria (unpublish observation) but when LysA is added from the outside .

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