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

Filipa Gil,1 Maria João Catalão,1 José Moniz-Pereira,1 Paula Leandro,2 Michael McNeil3 and Madalena Pimentel1

1Unidade dos Retrovírus e Infecções Associadas, Centro de Patogénese Molecular, Faculty of Pharmacy, University of Lisbon, Portugal
2Unidade de Biologia Molecular e Biopatologia Experimental, iMed, Faculty of Pharmacy, University of Lisbon, Portugal
3Department of Microbiology Immunology and Pathology, Colorado State University, Fort Collins, CO 80523, USA

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 λ cassette includes the rz 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 (lysA) and the holin (hol) genes, the Ms6 lytic cassette comprises three additional genes whose functions...
have not yet been identified. Between lysA 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), carboxyl-
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). Lipases
are, by definition, enzymes that have the ability to hydrolyse
long-chain acylglycerols (≥C₁₀), whereas esterases hydro-
lyse ester substrates with short-chain fatty acids (≤C₁₀)
(Arpigny & Jaeger, 1999; Jaeger et al., 1999; Bornscheuer,
2002). Cutinases hydrolyse the water-insoluble biopoly-
ester 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,
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 mycobac-
terio phages. 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 specifici-
ity, 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 (5′-GGCCACTGGATCCCCGATGACG-3′) and
PrLys3C (5′-GCATGGTGACCTAACCTGATCGGC-3′), using
Ms6 DNA as a template. The PCR product was purified with the
MinElute PCR Purification kit (Qiagen), digested with BamHI and
SalI, 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
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

Expression and purification of LysB protein. E. coli JM109
(pMP302) was grown in LB medium to an OD₆₀₀ of 0.6, and
expression of the recombinant His₅-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 His₅-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 His₅-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 CaCl₂, or (d) 1 % (v/v) Tween 20 with 1 mM CaCl₂
(Kouker & Jaeger, 1987; Nikoleit et al., 1995). A 100 μg quantity of
His₅-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 UV lamp on triolein plates, and by
the formation of a white precipitate on Tween plates. For the specific
detection of lipase activity, 100 μg His₅-LysB was incubated at room
temperature with 100 μg 1,2-Dilauryl-rac-glycerol-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 (ΔA₅₇₂ min⁻¹)
over a period of 10 min (Schmidt et al., 2004). The hydrolase
activity of recombinant His₅-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 His₅-LysB
(100 μg).

The substrate specificity of the purified His₅-LysB was measured using
pNP esters (Sigma) with carbon chain length ranging from C₄ to C₁₈.
The released pNP from the substrates pNP ester (Sigma) with carbon
chain length ranging from C₄ to C₁₈ was measured using a spectrophotometric assay. The enzymic reaction
was performed at room temperature (25 °C) in a final volume of
200 μl containing 60 μg purified His₅-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

http://mic.sgmjournals.org
were performed as described above using substrate concentrations ranging from 10 μM to 5 mM.

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 His6-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 His6-LysB, the enzymic assay was performed at standard conditions using pNPB as the substrate, in the presence of one of the following metal ions (5 mM): Ca2+, Mg2+, K+, Cd2+, Mn2+, Hg2+ and Zn2+. 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.

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 His6 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 % CHCl₃ (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 His6-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 His6 tag on recombinant His6-LysB (Fig. 1).

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 lysin 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 show 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 fungi. 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).

**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 His₆-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 (C₁₈) and lauric (C₁₂) acids, respectively, and can be cleaved by lipolytic enzymes to produce a fatty acid and an alcohol. The presence of Ca²⁺ 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 His₆-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 His₆-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-O-dilauryl-rac-glycero-3-glutaric acid resorufin ester (Jaeger et al., 1999; Schmidt et al., 2004). As can be observed in Fig. 4, His₆-LysB was able to hydrolyse this substrate releasing...
resorufin (Fig. 4) with a ΔA572 of approximately 0.14 min⁻¹, confirming that His6-LysB has lipase activity. The enzymic activity of recombinant His6-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 His6-LysB does not have proteolytic or haemolytic activity.

Biochemical characterization of LysB lipase

The activity of His6-LysB was measured using pNP esters of different chain lengths. As shown in Fig. 5, His6-LysB was able to hydrolyse all the tested substrates, showing the highest activity (0.12 U mg⁻¹) with the short-chain pNPB (C₄). In order to examine the specificity of His6-LysB towards the length of the acyl chain, the kinetic parameters of the enzyme were determined. The rates of hydrolysis of different concentrations of all substrates were measured, and the substrate affinity constant (K₀.₅) and the turnover of the enzyme reaction (Kcat) were obtained, and are shown with the deduced catalytic efficiency (Kcat/K₀.₅) (Table 1). The K₀.₅ values had a tendency to decrease with the increase in the chain length of substrates. The short-chain pNPB showed the highest Kcat and K₀.₅ values, resulting in a low catalytic efficiency (0.61 min⁻¹ μM⁻¹). Although the highest affinity was observed for the long-chain pNPP and pNPS, 145 μM and 146 μM respectively, pNPS presented a lower catalytic efficiency.

**Table 1.** Kinetic parameters of recombinant LysB for the hydrolysis of pNP esters of different chain lengths (C₄–C₁₈) as substrates

<table>
<thead>
<tr>
<th>pNP ester</th>
<th>Kcat (min⁻¹)*</th>
<th>K₀.₅ (μM)*</th>
<th>Kcat/K₀.₅ (min⁻¹ μM⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pNPB (C₄)</td>
<td>449 ± 26</td>
<td>726 ± 24</td>
<td>0.61</td>
</tr>
<tr>
<td>pNPC (C₆)</td>
<td>247 ± 20</td>
<td>348 ± 1</td>
<td>0.71</td>
</tr>
<tr>
<td>pNPL (C₁₂)</td>
<td>297 ± 38</td>
<td>260 ± 30</td>
<td>1.14</td>
</tr>
<tr>
<td>pNPM (C₁₄)</td>
<td>278 ± 32</td>
<td>246 ± 3</td>
<td>1.13</td>
</tr>
<tr>
<td>pNPP (C₁₆)</td>
<td>146 ± 0.32</td>
<td>145 ± 8</td>
<td>1.07</td>
</tr>
<tr>
<td>pNPS (C₁₈)</td>
<td>117 ± 3.2</td>
<td>146 ± 34</td>
<td>0.80</td>
</tr>
</tbody>
</table>

*Values are means ± sd from three independent determinations.
(0.80 min⁻¹ μM⁻¹) than pNPP (1.07 min⁻¹ μM⁻¹). 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).

Effect of different metal ions and inhibitors

The effect of different metal cations on the activity of His₆-LysB was assayed using pNPB as the substrate. Table 2 indicates that the presence of Ca²⁺ or Mn²⁺ increased the activity of the recombinant protein; this was seen especially with Ca²⁺ (5 mM), which increased the activity by 99.2 %. Mg²⁺ and K⁺ made no significant difference on the activity of the enzyme, while Zn²⁺, Cd²⁺ and Hg²⁺ caused a reduction in His₆-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 2). 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.

**Table 2. Effect of different metal ions and inhibitors on His₆-LysB activity using pNPB as the substrate**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (mM)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca²⁺</td>
<td>5</td>
<td>199.2 ± 2.5</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>5</td>
<td>108.8 ± 0.9</td>
</tr>
<tr>
<td>K⁺</td>
<td>5</td>
<td>99.2 ± 2.0</td>
</tr>
<tr>
<td>Cd²⁺</td>
<td>5</td>
<td>3.4 ± 0.8</td>
</tr>
<tr>
<td>Hg²⁺</td>
<td>5</td>
<td>6.5 ± 0.4</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>5</td>
<td>136.1 ± 0.9</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>5</td>
<td>6.2 ± 0.5</td>
</tr>
<tr>
<td>EDTA</td>
<td>10</td>
<td>114.4 ± 3.6</td>
</tr>
<tr>
<td>PMSF</td>
<td>10</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>DEPC</td>
<td>20</td>
<td>54.1 ± 0.3</td>
</tr>
</tbody>
</table>

*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.

**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 lysA, 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. His₆-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-O-dilauryl-rac-glycero-3-gluatrlic 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 His6-LysB displayed activity towards substrates with chain lengths from C4 to C18, it showed a higher affinity for pNP esters of longer chain length (C16 and C18). These results, together with the higher catalytic efficiency revealed for C12 and C14 pNP esters, indicate that the long-chain substrates must represent the natural substrate of His6-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 pentapeptide (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 of Mycobacterium smegmatis. Alignment of these proteins showed that the characteristic pentapeptide 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 His6-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, His6-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). Although three Asp residues (Asp215, Asp249 and Asp306 on LysB amino acid sequence) are totally conserved, Asp215 together with His246 might be good candidates for the catalytic triad obeying the order Ser-Asp-His. Further experiments will be needed to confirm this hypothesis. 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. His6-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 His6-LysB. The activity of the enzyme was affected by the presence of metal ions. It is possible that Zn2+, Cd2+ and Hg2+ affect the catalytic site directly, since incubation of the recombinant His6-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). Hg2+ is a thiol-reactive agent, and its ability to compromise His6-LysB activity suggests that thiol groups compromise His6-LysB activity. This in turn indicates that His residues participate in the active site of the enzyme. Ca2+ 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 arabinoaraboglucan polysaccharide, which is attached to the peptidoglycan backbone. This mycolyl–arabinoaraboglucan–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, Mycobacterium tuberculosis, for which new therapeutic alternatives are urgently needed.

**ACKNOWLEDGEMENTS**

This work was supported by funds provided by FCT (Fundação para a Ciência e Tecnologia) PTDC/SAU-FCF/73017/2006. Filipa Gil is the recipient of a fellowship from FCT (SFRH/BD/29167/2006), and
REFERENCES


Garcia, M., Pimentel, M. & Moniz-Pereira, J. (2002). Expression of mycobacteriophage Ms6 lysis genes is driven by two sigma (70)-like promoters and is dependent on a transcription termination signal present in the leader RNA. J Bacteriol 184, 3034–3043.


Edited by: J. Anné

Maria João Catalão is the recipient of a fellowship from FCT (SFRH/BD/24452/2005).