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

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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 His₆-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 (C₄–C₁₈), it presents a higher affinity for *p*-nitrophenyl esters of longer chain length (C₁₆ and C₁₈). 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 Ca²⁺ and Mn²⁺. To the best of our knowledge, this is the first description of a protein with lipolytic activity encoded within a bacteriophage.

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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

Abbreviations: DEPC, diethylpyrocarbonate; 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.

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), 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 ($\geq C_{10}$), whereas esterases hydrolyse ester substrates with short-chain fatty acids ($\leq C_{10}$) (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 (5'-GGGCCACTGGATCCCGCATCGACG-3') and PrLys3C (5'-GCATGGGTGACCTCCTATGTGCG-3'), 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 OD₆₀₀ of 0.6, and expression of the recombinant His₆-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 His₆-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 His₆-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 His₆-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 His₆-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 ($\Delta A_{572} \text{ min}^{-1}$) over a period of 10 min (Schmidt *et al.*, 2004). The hydrolase activity of recombinant His₆-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 His₆-LysB (100 µg).

The substrate specificity of the purified His₆-LysB was measured using pNP esters (Sigma) with carbon chain length ranging from C₄ to C₁₈. The released pNP from the substrates *p*-nitrophenyl butyrate (pNPB; C₄), *p*-nitrophenyl caprylate (pNPC; C₈), *p*-nitrophenyl laurate (pNPL; C₁₂), *p*-nitrophenyl myristate (pNPM; C₁₄), *p*-nitrophenyl palmitate (pNPP; C₁₆) and *p*-nitrophenyl stearate (pNPS; C₁₈) 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 His₆-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 μ M 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 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 μ mol 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 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

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

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 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.

LysB	Ms6	93	PVLFTVCGTGVPPWVGP--DADTARAVEDQYLWQPIG--YPAAPFFMGRSI	139
Gp33	Che8	94	PVLITVCGTGVPPWIGP--DADTARAVEDKYLWQPIG--YPAAPFFMGKSI	140
Gp70	Corndog	101	PLFITVCGTGVPPWVGP--DADTARACEDKLFWQPIG--YPAKNFFMGKSI	147
Gp238	Bxz1	98	PVLFTVCGTGVPPWVGP--DAETARQVEDLYRWQPIG--YPAAPFFMGPSI	144
Gp9	Bxb1	81	PVLLTVSGTGVPPWVGP--DADVARRLGDVYLWRPVGPPYTAQAFPMGSPV	129
Gp52	Wildcat	86	PTLYTVHGTGVSMWDGP--PADCARRLLDKYRWQPIG--NYPASAFPMWPSI	133
Gp12	L5	4	PWLFTVHGTGQPDPLGGLPADTARDVLDIYRWQPIG--NYPAAAFPMWPSV	53
Gp12	Bxz2	83	RWLFTVHGTGQADPLGGLPADTARAVLDKYTWQPIG--NYPARAFPMWSSI	132
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LysB	Ms6	154	RERIETHGTALAGYSQGA	177
Gp33	Che8	155	RERIETHGAALAGYSQGA	178
Gp70	Corndog	168	RQRIERNGVVLGGYSQGA	191
Gp238	Bxz1	165	RKQVERNGMVLGYSQGA	188
Gp9	Bxb1	144	RRRIERYGLSMIGYSQGA	167
Gp52	Wildcat	157	----TPGKFTLAGYSQGA	176
Gp12	L5	68	LDADPYADFALAGYSQGA	91
Gp12	Bxz2	157	S-----GEVNLAGYSQGA	165
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LysB	Ms6	241	WRDYAHQGDLYACTEPGDTQEVNRN	303
Gp33	Che8	242	WRDYAHQGDLYACTEPGDTQEVNRN	304
Gp70	Corndog	255	WRNYAHAGDLYAAAEFGDSQDKNAIWQI	317
Gp238	Bxz1	251	WRNYAHQGDLYTATRPGESREDKVAIWQI	313
Gp9	Bxb1	230	WRNYAHKGDLYTDCE-GDSGEMKTAIYKVVMSRVFS	291
Gp52	Wildcat	229	WRDYAHKGDLYTDCEFDDEGEYKRSVCKI	291
Gp12	L5	158	VRDYAHGDMDYASIKEDDMHEYEVAIGRIVMS	221
Gp12	Bxz2	232	IRDYAHAGDMYASITDGDKEYKIAICKIVMTATDFYRGPN	295
*:*** **:*: . : . : : : : * :*: * *: * . * *				
LysB	Ms6		ILDAGMFFAKRTG---PHVDYNPQPAIDYLR	332
Gp33	Che8		LLDAGMFFAKRTG---PHVDYNVQPAIDYLR	333
Gp70	Corndog		LIDAGMFFAKGTG---PHVDYIGGPAIEYLR	346
Gp238	Bxz1		FMDAGLFFVKGTR---PHTNYHIGAAYDYLR	342
Gp9	Bxb1		VLDAGLFFIRGTG---PHVNYNIDPATDFLR	322
Gp52	Wildcat		ISDAGMFFINRTT---PHINYNVGPAVDFLAGL	321
Gp12	L5		IIDALTFFAKSTQGPSWPHLYNRFPVDFLR	254
Gp12	Bxz2		IIDTLRFFNTAHG-----YNIGPAIDFLR	321
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Fig. 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.

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 (trioline 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

Fig. 3. Effect of His₆-LysB activity on agar plates containing triolein and rhodamine B, tributyrin, Tween 20 and CaCl₂, and Tween 80 and CaCl₂. The plates were incubated with 100 µg purified His₆-LysB for at least 24 h at 37 °C.

resorufin (Fig. 4) with a ΔA_{572} 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.

Fig. 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.

Fig. 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 $1 \text{ µmol p-NP min}^{-1}$.

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 ($K_{0.5}$) and the turnover of the enzymic reaction (K_{cat}) were obtained, and are shown with the deduced catalytic efficiency ($K_{\text{cat}}/K_{0.5}$) (Table 1). The $K_{0.5}$ values had a tendency to decrease with the increase in the chain length of substrates. The short-chain pNPB showed the highest K_{cat} and $K_{0.5}$ values, resulting in a low catalytic efficiency ($0.61 \text{ min}^{-1} \text{ µM}^{-1}$). 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

pNP ester	K_{cat} (min^{-1})*	$K_{0.5}$ (µM)*	$K_{\text{cat}}/K_{0.5}$ ($\text{min}^{-1} \text{ µM}^{-1}$)
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 $\pm$ SD from three independent determinations.

($0.80 \text{ min}^{-1} \mu\text{M}^{-1}$) than pNPP ($1.07 \text{ min}^{-1} \mu\text{M}^{-1}$). The highest catalytic efficiency was observed with the relatively long-chain-length pNPL and pNPM, with catalytic efficiencies of 1.14 and $1.13 \text{ min}^{-1} \mu\text{M}^{-1}$, 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

Table 2. 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.

Fig. 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.

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 metallo-hydrolase. 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.

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-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 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 *M. smegmatis* or the pathogenic *Mycobacterium tuberculosis*. 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 may be important for catalysis (Teo *et al.*, 2003). In contrast, Ca²⁺ and Mn²⁺ exhibited a stimulatory effect on the enzyme activity. 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 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.

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