

Isolation and characterization of *Bacillus* sp. BP-6 LipA, a ubiquitous lipase among mesophilic *Bacillus* species

C. Ruiz, F.I. Javier Pastor and P. Diaz

Department of Microbiology, Faculty of Biology, University of Barcelona, Barcelona, Spain

2003/0244: received 21 March 2003, revised 23 July 2003 and accepted 26 July 2003

ABSTRACT

C. RUIZ, F.I. JAVIER PASTOR AND P. DIAZ. 2003.

Aims: The aim of this study was to perform the isolation, cloning and characterization of a lipase from *Bacillus* sp. BP-6 bearing the features of a biotechnologically important group of enzymes.

Methods and Results: Strain *Bacillus* sp. BP-6, showing activity on tributyrin plates, was used for isolation of lipase-coding gene *lipA* by means of inverse and direct PCR. The complete 633 nucleotide ORF isolated was cloned in *Escherichia coli* for further characterization. The amino acid sequence of the cloned protein was 98% identical to *B. subtilis* and *B. megaterium* lipases, the enzyme also showing similar molecular and biochemical features.

Conclusions: The gene coding for *Bacillus* sp. BP-6 LipA was found in all mesophilic *Bacillus* species assayed, indicating its ubiquity in the genus. The cloned enzyme displayed the same properties as those of homologous lipases.

Significance and Impact of the Study: The overall profile of *Bacillus* sp. BP-6 LipA was found to be that of a ubiquitous and highly conserved subfamily I.4 bacterial lipase. Previously described lipases within this family have shown to be well suited for biotechnological applications, suggesting that the cloned enzyme could be used accordingly.

Keywords: *Bacillus*, carboxylesterase, lipase.

INTRODUCTION

Lipases and esterases (EC 3.1.1.–) are a diverse group of enzymes that catalyse the hydrolysis of ester bonds in triacylglycerides, showing activity on a great variety of substrates (Jaeger *et al.* 1999). These enzymes contain a catalytic triad that usually consists of a serine, a histidine and an aspartic acid, with the serine embedded in the consensus sequence G-X-S-X-G at the active site (Wang and Hartsuck 1993). Ester hydrolysis is mediated by a nucleophilic attack of the active serine on the carbonyl group of the substrate, in a charge-relay system with the two other amino acid residues of the catalytic triad (Jaeger *et al.* 1999). In nonaqueous systems, lipases catalyse the reverse reaction,

namely ester synthesis and transesterification. They can also catalyse stereoselective and regioselective reactions (Jaeger *et al.* 1999). Therefore, there is an increasing interest for lipases and lipase-producing strains for biotechnological applications, as lipase-catalysed reactions show high selectivity and occur under mild conditions, with no requirement for added cofactors (Schmidt-Dannert 1999).

Bacillus species are among those micro-organisms capable to promote lipid conversion by means of their lipolytic systems. Several lipases from *B. subtilis* (Dartois *et al.* 1992; Eggert *et al.* 2000), *B. pumilus* (Moeller *et al.* 1992), *B. licheniformis* (Nthangeni *et al.* 2001), *B. megaterium* (Ruiz *et al.* 2002), *B. thermocatenulatus* (Schmidt-Dannert *et al.* 1996), *B. thermoleovorans* (Lee *et al.* 1999), or *B. stearothermophilus* (Kim *et al.* 1998) have already been described, cloned or purified. Among them, the small secreted *B. subtilis* lipases LipA (Dartois *et al.* 1992) and LipB (Eggert *et al.*

Correspondence to: Pilar Diaz, Department of Microbiology, Faculty of Biology, University of Barcelona, Av. Diagonal 645, 08028 Barcelona, Spain (e-mail: pdiaz@bio.ub.es).

2000) have been shown to be well suited for biotechnological applications (Jaeger *et al.* 1999).

Bacterial lipases and esterases have been grouped into eight sequence homology families (Arpigny and Jaeger 1999), where the small (19 kDa) *B. subtilis* LipA (Dartois *et al.* 1992) and LipB (Eggert *et al.* 2000) were grouped into subfamily I.4 of bacterial true lipases (EC 3.1.1.3), sharing 75% identity with lipases from the moderately mesophilic *B. pumilus* and *B. licheniformis* (Arpigny and Jaeger 1999), and 98% identity to *B. megaterium* LipA (Ruiz *et al.* 2002), all of them belonging to the same subfamily.

A significant rate of sequence homology has frequently been found among genes coding for similar functions in *B. subtilis* and other *Bacillus* species (Prim *et al.* 2001), suggesting that genes coding for the same type of proteins in different species of this genus could display stretches of sequence identity. The moderately mesophilic character of strains *Bacillus* sp. BP-6 and *Bacillus* sp. CR-179, and the presence of *ca* 20 kDa protein band, respectively, with activity on methylumbelliferone butyrate lead us to perform the isolation of the corresponding coding genes by means of molecular techniques. Thus, alignment of the conserved stretches of amino acids found among previously described *Bacillus* lipases allowed the isolation of a complete ORF from *Bacillus* sp. BP-6, coding for LipA. The cloning strategy and the characterization of the encoded lipase in comparison with the general properties described for other *Bacillus* lipases of biotechnological interest (Eggert *et al.* 2000; Ruiz *et al.* 2002) are presented here.

MATERIALS AND METHODS

Strains, plasmids and growth conditions

Bacillus sp. BP-6 and *Bacillus* sp. CR-179, two strains isolated from different habitats (Spain and Argentina, respectively) were grown in nutrient broth at 30 and 42°C. *Escherichia coli* 5K, used as the recipient strain for recombinant plasmids, was grown in Luria-Bertani medium at 37°C. Plasmid pBR322 (Boehringer Mannheim, Mannheim, Germany) was used as cloning vector. Lipolytic activity was detected on agar plates supplemented with 1% tributyrin and 0.0002% Rhodamine B (Kouker and Jaeger 1987), or by 4-methylumbelliferone (MUF) release from MUF-derivative substrates (Diaz *et al.* 1999).

Nucleic acid manipulation

The DNA was purified and manipulated essentially as described (Sambrook *et al.* 1989). Primers were purchased at Difco BRL, and *pfu* polymerase was from Stratagene (La Jolla, CA, USA). PCR was usually performed using 29 cycling periods of 30 s at 94°C, 40 s at 50°C, and 4 min at

72°C. Amplified DNA was purified through WIZARD® columns (Promega, Madison, WI, USA) and sequenced as described (Prim *et al.* 2000). Homology was analysed through BLAST (Altschul *et al.* 1997), sequence alignments were performed using ClustalW (1.74) Multalign program, and signal peptide identification was performed through SignalP V2.0 software (Nielsen *et al.* 1997). The physico-chemical parameters of the deduced amino acid sequences were analysed at ExPASy (<http://www.expasy.org>).

Cloning procedure

Known lipase sequences from mesophilic *Bacillus* species (M74010, D78508, A34992, AJ297356, AF232707 and AJ430831) were aligned, the stretches of sequence homology identified, and a set of primers – FWSUB (5'-GAT ATT GTG GCT CAT AGT ATG GGC GG), BKSUB (5'-GGC CTC CGC CGT TCA GCC CTT C) – designed for amplification of lipase-coding DNA fragments from *Bacillus* sp. BP-6 or *Bacillus* sp. CR-179 chromosomal DNA. A new set of specific divergent primers – BKBP6 (5'-CCC ACC GAG CGT GAC AAC ATT TTG), FWBP6 (5'-AGC CAA GTC AAC GCC TAT ATC AAA G) – was designed for sequencing and isolation of the complete lipase-coding gene from *Bacillus* sp. BP-6, by means of inverse and direct PCR (Ruiz *et al.* 2002). The isolated complete ORF was ligated to *Eco* RV-digested pBR322, and cloned in *E. coli* 5K for further characterization (Ruiz *et al.* 2002).

Activity assays

Activity and zymogram assays were routinely performed using crude cell extracts or concentrated culture supernatants, prepared as described before (Ruiz *et al.* 2002). The release of *para*-nitrophenol (*p*NP) or 4-MUF from *p*NP or MUF-derivative substrates was measured as described (Prim *et al.* 2000). One unit of activity was defined as the amount of enzyme that released 1 µmol of *p*NP or MUF per minute under the assay conditions used. Electrophoresis and isoelectric focusing (IEF) were performed as previously described (Prim *et al.* 2000). After protein separation, activity was detected by zymogram, and gels were subsequently stained with Coomassie Brilliant Blue R®-250 for protein band visualization (Diaz *et al.* 1999).

Nucleotide sequence accession number

The DNA sequence of *Bacillus* sp. BP-6 (*lipA*_BACBP6) was submitted to the EMBL under accession number AJ430985.

RESULTS

Screening for lipolytic strains

Several bacterial strains isolated either from Ebro's delta river in Spain or from Iguazú falls in Argentina were screened for lipolytic activity (Prim *et al.* 2000; Ruiz *et al.* 2002). Two strains, *Bacillus* sp. BP-6 (from Spain) and *Bacillus* sp. CR-179 (from Argentina), produced large hydrolysis haloes when analysed on tributyrin plates (not shown), indicating that both strains coded for at least one lipolytic enzyme.

Sequence analysis

Two lipase-coding genes were isolated as described in Materials and Methods from both *Bacillus* strains, and their corresponding sequences analysed. In both cases, a 633 nucleotide DNA fragment coded for an identical predicted protein of 210 amino acids with a deduced molecular mass of 22 331 Da, and a pI of 9.43. A 28-residue typical *Bacillus* signal peptide was found at the N-terminal region of both proteins, indicating their extracellular location. The common motif A-H-S-M-G of all known mesophilic *Bacillus* lipases was found in both proteins, with the first glycine typical residue (Wang and Hartsuck 1993) being replaced by an alanine (Jaeger *et al.* 1999). The catalytic apparatus of lipases, involving the triad serine, glutamate or aspartate, and histidine (Wang and Hartsuck 1993) was placed in the newly isolated lipases at positions S⁷⁸, D¹³⁴ and H¹⁵⁷. Both proteins showed an identical amino acid sequence, which was 98% identical to that of *B. subtilis* LipB (Yamamoto *et al.* 1996; Eggert *et al.* 2000) or *B. megaterium* LipA (Ruiz *et al.* 2002), and 74–72% homologous to those of *B. subtilis* LipA (Dartois *et al.* 1992) or *B. pumilus* lipase (Moeller *et al.* 1992). As a result of the identity of the two isolated lipases, only strain *Bacillus* sp. BP-6 was further characterized in this study.

Analysis of *Bacillus* sp. BP-6 lipolytic system

The activity of concentrated supernatants and cell extracts from *Bacillus* sp. BP-6 was analysed on SDS-PAGE and IEF gels, using MUF-butyrate as a substrate (Diaz *et al.* 1999). A faint activity band (not shown) with the size of the predicted mature lipase (*ca* 19 kDa) was observed in concentrated culture supernatants of the strain. A single band with the corresponding predicted pI (9.2) could also be appreciated in IEF gel zymograms (Fig. 1), suggesting that *Bacillus* sp. BP-6 produces only one extracellular lipase, named LipA, with activity on MUF-butyrate. SDS-PAGE zymographic analysis of *Bacillus* sp. BP-6 crude cell extracts showed the presence of several activity bands with sizes ranging from 110 to 27 kDa (Fig. 1). However, when

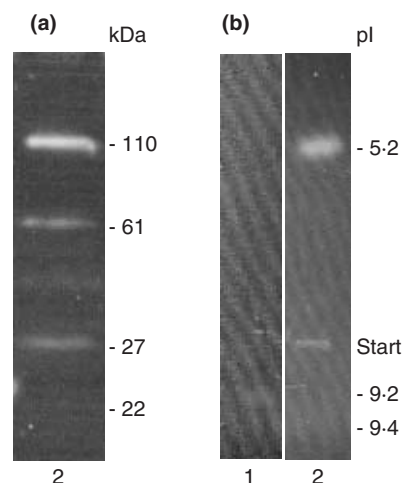


Fig. 1 SDS-PAGE and IEF zymograms on MUF-butyrate of *Bacillus* sp. BP-6 lipases. (a) Zymogram analysis of cell extracts from *Bacillus* sp. BP-6 (2) separated by SDS-PAGE. (b) IEF zymogram of *Bacillus* sp. BP-6 concentrated supernatants (1) and cell extracts (2). The molecular mass and pI of the bands are indicated. An unspecific activity band appears at the application point (Start) of concentrated cell extracts on the IEF gels

cell extracts of this strain were analysed by isoelectrofocusing, a single activity band with a pI of 5.2 could be detected, suggesting that the multiple bands appearing at SDS-PAGE could be the result of aggregation, a phenomenon frequently described for lipolytic enzymes (Prim *et al.* 2000).

Cloning and performance of *Bacillus* sp. BP-6 extracellular LipA

The isolated DNA fragment from *Bacillus* sp. BP-6 was cloned in *E. coli* 5K and the resulting recombinant clone, *E. coli*/pBP6, further characterized. Clear hydrolysis zones were detected after growth on agar plates supplemented with Rhodamine B and tributyrin (Kouker and Jaeger 1987), whereas poor degradation was found on plates containing triolein or olive oil, the typical substrates for true lipases (Jaeger *et al.* 1999).

Cell extracts from the recombinant clone were tested for lipolytic activity on several pNP and MUF derivatives. As shown in Table 1, the cloned enzyme efficiently hydrolysed esters of short-chain fatty acids, whereas activity decreased as the length of the fatty acid chains increased, a behaviour similar to that described for esterases (Jaeger *et al.* 1999). When the substrate profile was tested on MUF-derivative substrates, maximum activity was also found on MUF-butyrate (Table 1).

Table 1 Substrate profile of *Bacillus* sp. BP-6 Lipase A

Substrate	Activity (U per mg protein)
<i>p</i> NP-butyrate	1.410
<i>p</i> NP-valerate	1.040
<i>p</i> NP-caproate	0.810
<i>p</i> NP-caprylate	0.320
<i>p</i> NP-caprate	0.080
<i>p</i> NP-laurate	0.026
<i>p</i> NP-palmitate	0.011
<i>p</i> NP-stearate	0.002
MUF-butyrate	0.040
MUF-oleate	0.004

Activity values are the mean of three independent assays using recombinant *Escherichia coli* 5K cell extracts.

Enzyme characterization

The effect of pH and temperature on the activity of *Bacillus* sp. BP-6 LipA was determined using MUF-butyrate as a substrate. The highest activity was found at 45°C, pH 7.0, exhibiting 87 and 66% residual activity at pH 6.0 and pH 9.0, respectively. The enzyme was stable in a pH range from 4.0 to 12.0 when incubated for 1 h at room temperature. The cloned lipase remained stable at temperatures ranging from 4 to 30°C when incubated at pH 7.0 for a period of 40 days. However, a dramatic decrease of activity (9.1% residual activity) was observed after incubation for 1 h at temperatures above 50°C.

The effect of different agents on the activity of the cloned lipase was determined using *p*NP-laurate (Table 2). Hg²⁺ caused high inhibition, while Ag⁺ led to a significant reduction of activity. Partial inhibition was caused by 10 mM concentrations of Fe³⁺, Cu²⁺, Pb²⁺ and Zn²⁺, whereas the rest of ions and concentrations assayed did not significantly affect the activity of the cloned enzyme. The influence of amino acid modifiers such as *p*-hydroxy-mercury-benzoic acid (PHMB, cysteine), N-acetyl-imidazol (NAI, tyrosine), and N-bromosuccinimide (NBS, tryptophan) was also tested (Table 2). Only NBS caused a significant reduction of activity, suggesting that tryptophan, but not cysteine nor tyrosine are involved in the functional or structural domains of LipA. In agreement with previous data on lipolytic enzyme inactivation (Prim *et al.* 2000; Ruiz *et al.* 2002), high inhibition was caused by urea, phytic acid and phenylmethylsulphonyl fluoride (Table 2), while activity remained more stable in the presence of EDTA, SDS or Triton X-100. The results obtained are consistent with the presence of a serine residue at the active site of the cloned lipase (Jaeger *et al.* 1999).

Coomassie stained gels of crude cell extracts from *E. coli* 5K bearing *Bacillus* sp. BP-6 LipA did not show the

Table 2 Effect of different agents on *Bacillus* sp. BP-6 Lipase A

Agent	Activity (%)	
	1 mM	10 mM
Control	100.0	100.0
CaCl ₂	96.4	88.1
FeCl ₂	110.1	46.9
NiCl ₂	92.0	99.5
CoCl ₂	96.1	98.8
ZnCl ₂	100.4	82.0
BaCl ₂	104.6	108.3
MnCl ₂	102.3	85.6
AgNO ₃	45.9	35.0
Pb acetate	102.2	83.2
CuSO ₄	91.2	71.9
MgCl ₂	102.0	103.6
NH ₄ Cl	103.2	119.5
HgCl ₂	26.3	18.2
NAI	105.8	84.0
PHMB	109.9	120.5
NBS	63.7	29.9
EDTA	110.1	75.8
PMSF	10.2	5.2
SDS (0.4%)	115.6	–
Triton X-100 (1.0%)	89.2	–
Urea (2 M)	55.0	–
Phytic acid (0.32%)	2.9	–

Activity values are the mean of three independent assays on *p*NP-laurate using recombinant *E. coli* 5K cell extracts.

presence of any additional band to those detected in control extracts from *E. coli* 5K/pBR322 (Fig. 2). Nevertheless, when analysed as zymograms, cell extracts of the recombinant clone showed the presence of a prominent band that hydrolysed MUF-butyrate, both in SDS and IEF gels, not present in control *E. coli* 5K/pBR322 (not shown). The size (*ca* 22 kDa) and *pI* (9.4) of these activity bands correspond to those deduced for the unprocessed form of LipA.

DISCUSSION

The presence of large hydrolysis haloes after incubation of strains *Bacillus* sp. BP-6 and *Bacillus* sp. CR-179 on tributyrin plates indicates that both strains code for at least one lipolytic enzyme. After specific PCR amplification, both strains produced a DNA fragment coding for an identical deduced protein bearing the typical motives of secreted *Bacillus* lipases (Dartois *et al.* 1992; Eggert *et al.* 2000). The results obtained from amino acid sequence homology analysis indicate that both lipases are members of a reduced cluster of highly conserved bacterial serine-esterases grouped in family I.4, exclusive from the mesophilic or moderately thermophilic members of the genus *Bacillus*

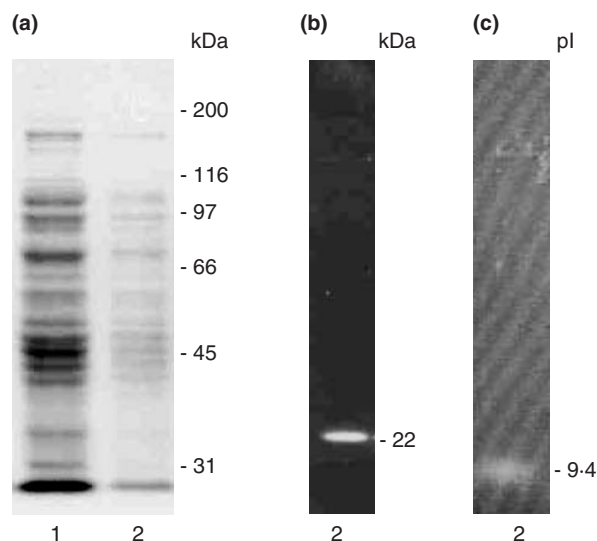


Fig. 2 Analysis of cloned *Bacillus* sp. BP-6 LipA. Coomassie stain (a) and zymograms on MUF-butyrate (b, c) of cell extracts from *E. coli* 5K/pBR322 (1) and recombinant clone *E. coli* 5K/pBP6 (2), analysed by SDS-PAGE (b) and IEF (c). The molecular mass and pI of the bands are indicated

(Arpigny and Jaeger 1999). The finding of such a well-conserved gene in all studied *Bacillus* mesophilic species, included those from unrelated habitats like *Bacillus* sp. CR-179, suggests that the encoded lipase must play an essential role in lipid transformation in nature.

Zymographic analysis of supernatants and cell extracts from strain *Bacillus* sp. BP-6 indicated that a single lipase with activity on MUF-butyrate and a molecular mass similar to *B. subtilis* LipA and LipB (Dartois *et al.* 1992; Eggert *et al.* 2000) was secreted to the external medium. An additional lipolytic enzyme was found in cell extracts of the strain. The size and pI similarity of *Bacillus* sp. BP-6 intracellular activity band with those of previously described bacterial carboxylesterases, suggests that this strain could bear a cell-bound esterase similar to those found in other Gram-positive bacteria (Zock *et al.* 1994; Prim *et al.* 2000).

The secreted LipA from *Bacillus* sp. BP-6 was cloned in *E. coli* and its properties analysed. The cloned enzyme displayed a substrate profile similar to that found for most esterases, showing higher affinity for short chain-length substrates (Jaeger *et al.* 1999). These results were confirmed after analysis of the kinetic parameters of the cloned enzyme on MUF-butyrate and MUF-oleate. On both substrates, a standard Michaelis–Menten plot was obtained (not shown), with apparent constants of 0.03 mM for MUF-butyrate and 0.048 mM for MUF-oleate. No interfacial activation was observed and, according to the proposed model for esterases and true lipases (Jaeger *et al.* 1999), the cloned enzyme

showed the kinetics of an esterase, very similar to *B. subtilis* LipB and *B. megaterium* LipA (Eggert *et al.* 2000; Ruiz *et al.* 2002), both grouped in subfamily I.4 of bacterial lipases.

The overall properties shown by *Bacillus* sp. BP-6 LipA indicate that the cloned lipase is a mesophilic enzyme acting on a broad range of pH that becomes inactivated only by a limited number of lipase inhibitors, as for most previously described lipases (Prim *et al.* 2000; Ruiz *et al.* 2002). The high homology found *B. subtilis* LipB (Eggert *et al.* 2000), *B. megaterium* LipA (Ruiz *et al.* 2002), and to the isolated lipase-coding gene from *Bacillus* sp. CR-179, indicate that the cloned LipA is highly ubiquitous and well conserved among mesophilic *Bacillus* species, where it probably plays an essential role in the transformation of lipids, independent of the habitat of the corresponding strains. Additionally, the high homology shown to *B. subtilis* lipases, a group of enzymes well suited for biotechnological applications (Jaeger *et al.* 1999), suggests that the cloned enzyme could be evaluated and used in certain manufacturing processes such as antibiotic transformation (Zock *et al.* 1994), biodegradation of recalcitrant substances (Pohlentz *et al.* 1992), or in the conversion of low-cost fats into added value products (Schmidt-Dannert 1999).

ACKNOWLEDGEMENTS

We thank Serveis Científico-Tècnics of the University of Barcelona for technical support in sequencing. This work was partially financed by the Scientific and Technological Research Council (CICYT, Spain), grant REN2001-3224, by the III Pla de Recerca de Catalunya (Generalitat de Catalunya), grant 2001SGR-00143, and by the Generalitat de Catalunya to the 'Centre de Referència en Biotecnologia' (CeRBa). C. Ruiz is a recipient of a fellowship from the Generalitat de Catalunya (2000FI-00187).

REFERENCES

- Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Gapped-BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**, 3389–3402.
- Arpigny, J.L. and Jaeger, K.E. (1999) Bacterial lipolytic enzymes: classification and properties. *Biochemical Journal* **343**, 177–183.
- Dartois, V., Baulard, A., Schanck, K. and Colson, C. (1992) Cloning, nucleotide sequence and expression in *Escherichia coli* of a lipase gene from *Bacillus subtilis* 168. *Biochimica et Biophysica Acta* **1131**, 253–260.
- Diaz, P., Prim, N. and Pastor, F.I.J. (1999) Direct fluorescence-based lipase activity assay. *BioTechniques* **27**, 696–700.
- Eggert, T., Pencreac'h, G., Douchet, I., Verger, R. and Jaeger, K.E. (2000) A novel extracellular esterase from *Bacillus subtilis* and its

- conversion to a monoacylglycerol hydrolase. *European Journal of Biochemistry* **267**, 6459–6469.
- Jaeger, K.E., Dijkstra, B.W. and Reetz, M.T. (1999) Bacterial biocatalysts: molecular biology, three-dimensional structures, and biotechnological applications of lipases. *Annual Reviews of Microbiology* **53**, 315–351.
- Kim, H.K., Park, S.Y., Lee, J.K. and Oh, T.K. (1998) Gene cloning and characterisation of thermostable lipase from *Bacillus stearothermophilus* L1. *Bioscience, Biotechnology and Biochemistry* **62**, 66–71.
- Kouker, G. and Jaeger, K.E. (1987) Specific and sensitive plate assay for bacterial lipases. *Applied and Environmental Microbiology* **53**, 211–213.
- Lee, D.W., Koh, Y.S., Kim, K.J., Kim, B.C., Choi, H.J., Kim, D.S., Suhartono, M.T. and Pyun, Y.R. (1999) Isolation and characterisation of a thermophilic lipase from *Bacillus thermoleovorans* ID-1. *FEMS Microbiology Letters* **179**, 393–400.
- Moeller, B., Vetter, R., Wilke, D. and Foullois, B. (1992) Alkane lipases from *Bacillus* and the cloning of their genes. Patent number WO9116422. Applicant(s) Kali Chemie A.-G.
- Nielsen, H., Engelbrecht, J., Brunak, S. and von Heijne, G. (1997) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Engineering* **10**, 1–6.
- Nthangeni, M.B., Patterton, H.G., Van Tonder, A., Vergeer, W.P. and Litthauer, D. (2001) Over-expression and properties of a purified recombinant *Bacillus licheniformis* lipase: a comparative report on *Bacillus* lipases. *Enzyme and Microbial Technology* **28**, 705–712.
- Pohlentz, H.D., Boidol, W., Schüttke, I. and Streber, W. (1992) Purification and properties of an *Arthrobacter oxydans* P52 carbamate hydrolase specific for the herbicide phenmedipham and nucleotide sequence of the corresponding gene. *Journal of Bacteriology* **174**, 6600–6607.
- Prim, N., Blanco, A., Martínez, J., Pastor, F.I.J. and Diaz, P. (2000) *estA*, a gene coding for a cell-bound esterase from *Paenibacillus* sp. BP-23, is a new member of the bacterial subclass of type B carboxylesterases. *Research in Microbiology* **151**, 303–312.
- Prim, N., Pastor, F.I.J. and Diaz, P. (2001) Cloning and characterisation of a bacterial cell-bound type B carboxylesterase from *Bacillus* sp. BP-7. *Current Microbiology* **42**, 237–240.
- Ruiz, C., Pastor, F.I.J. and Diaz, P. (2002) Analysis of *Bacillus megaterium* lipolytic system and cloning of LipA, a novel subfamily I.4 bacterial lipase. *FEMS Microbiology Letters* **217**, 263–267.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.
- Schmidt-Dannert, C. (1999) Recombinant microbial lipases for biotechnological applications. *Bioorganic and Medical Chemistry* **7**, 2123–2130.
- Schmidt-Dannert, C., Rúa, M.L., Atomi, H. and Schmid, R.D. (1996) Thermoalkalophilic lipase of *Bacillus thermocatenuatus*. I. Molecular cloning, nucleotide sequence, purification and some properties. *Biochimica et Biophysica Acta* **1301**, 105–114.
- Wang, C.S. and Hartsuck, J.A. (1993) Bile salt-activated lipase. A multiple function lipolytic enzyme. *Biochimica et Biophysica Acta* **1166**, 1–19.
- Yamamoto, H., Uchiyama, S. and Sekiguchi, J. (1996) The *Bacillus subtilis* chromosome region near 78° contains the genes encoding a new two-component system, three ABC transporters and a lipase. *Gene* **181**, 147–151.
- Zock, J., Cantwell, C., Swartling, J., Hodges, R., Pohl, T., Sutton, K., Rosteck, P., McGilvray, D. and Queener, S. (1994) The *Bacillus subtilis* *pnbA* gene encoding *p*-nitrobenzyl esterase: cloning, sequence and high-level expression in *Escherichia coli*. *Gene* **151**, 37–43.