ANNEXE I: PUBLICATIONS

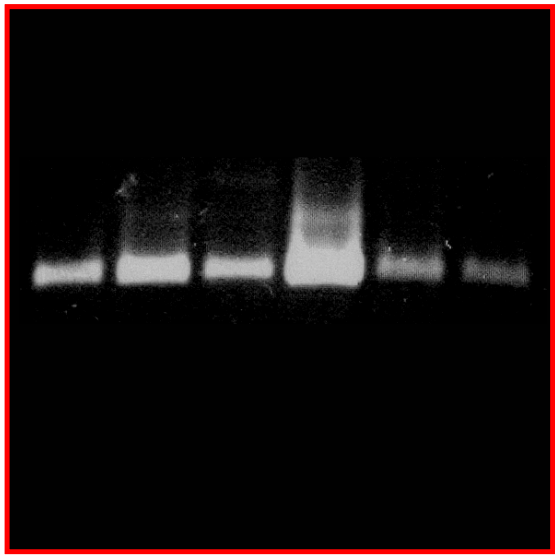

Figure A.4 Amplification of *B. subtilis lipB*-related genes.

Ruiz, C., Blanco, A., Pastor, F.I.J. and Díaz, P. (2002) Analysis of Bacillus megaterium lipolytic system and cloning of LipA, a novel subfamily I.4 bacterial lipase. *FEMS Microbiol. Lett.* 217: 263–267.

Prim, N., Sánchez M., Ruiz, C., Pastor, F.I.J. and Díaz, P. (2003) Use of methylumbeliferyl-derivative substrates for lipase activity characterization. *J. Mol. Catal. B Enzym.* 22: 339–346.

Ruiz, C., Pastor, F.I.J. and Díaz, P. (2003) Isolation and characterization of *Bacillus* sp. BP-6 LipA, a ubiquitous lipase among mesophilic *Bacillus* species. *Lett. Appl. Microbiol.* 37: 354–359.

Ruiz, C., Falcocchio, S., Xoxi, E., Pastor, F.I.J., Díaz, P. and Saso, L. (2004) Activation and Inhibition of *Candida rugosa* and *Bacillus*-related lipases by Saturated Fatty Acids, Evaluated by a New Colorimetric Microassay. *Biochim. Biophys. Acta* 1672: 184–191.

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Ruiz, C., Falcocchio, S., Xoxi, E., Villo, L., Nicolosi, G., Pastor, F.I.J., Díaz, P. and Saso, L. (2005) Inhibition of *Candida rugosa* lipase by saponins, flavonoids and alkaloids. *Biosci. Biotechnol. Biochem.* Submitted.

Ruiz, C., Falcocchio, S., Pastor, F.I.J., Saso, L. and Díaz, P. (2005) *Helicobacter pylori* 26695 ORF HP0739 codes for a previously unknown family V carboxylesterase. Cloning, purification, characterization and inhibition of the enzyme by natural substances. *J. Bacteriol.* Submitted.

Falcocchio, S., Ruiz, C., Pastor, F.I.J., Saso, L. and Díaz, P. *Propionibacterium acnes* GehA lipase: cloning, characterization and inhibition by natural substances. *In preparation*.

Ruiz, C., Falcocchio, S., Pastor, F.I.J., Saso, L. and Díaz, P. Characterization and inhibition of bacterial lipases involved in pathogenesis. *In preparation*.

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Analysis of *Bacillus megaterium* lipolytic system and cloning of LipA, a novel subfamily I.4 bacterial lipase

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Abstract

The lipolytic system of *Bacillus megaterium* 370 was investigated, showing the existence of at least two secreted lipases and a cell-bound esterase. A gene coding for an extracellular lipase was isolated and cloned in *Escherichia coli*. The cloned enzyme displayed high activity on short to medium chain length (C_4–C_8) substrates, and poor activity on C_{18} substrates. On the basis of amino acid sequence homology, the cloned lipase was classified into subfamily I.4 of bacterial lipases.

Keywords: *Bacillus*; *Bacillus megaterium*; Lipase; Carboxylesterase

1. Introduction

Lipases are a diverse group of enzymes that catalyse the hydrolysis of ester bonds in triacylglycerides, showing activity on a great variety of substrates. In non-aqueous systems, lipases catalyse the reverse reaction, namely ester synthesis and transesterification, and they can also direct stereoselective and regioselective reactions [1]. These versatile enzymes are therefore interesting biocatalists for industrial purposes, as most lipase-catalysed reactions show high selectivity and occur under mild conditions, with no requirement for added cofactors. These properties contribute to the lowering of costs in industrial conversions and justify the increasing interest for lipases and lipase-producing strains for biotechnological applications [2].

Among lipase-producing bacteria, several *Bacillus* species have been shown to possess a lipolytic system well suited for biotechnological applications [1], and lipases from *B. subtilis* [3–4], *B. pumilus* [5], *B. licheniformis* [6], *B. thermocatenulatus* [7], *B. thermoleovorans* [8], or *B. stearothermophilus* [9] have already been described, purified or cloned. The increasing interest for bacterial lipases and new lipase-producing *Bacillus* strains led us to perform the analysis of *Bacillus megaterium* 370 lipolytic system. Such study and the cloning of a novel extracellular lipase from the strain are reported here.

2. Materials and methods

2.1. Strains, plasmids and growth conditions

B. megaterium 370, a strain from the Spanish Type Culture Collection (CECT; ATCC9885) was routinely grown in nutrient broth at 30°C. *Escherichia coli* 5K [10], used as the recipient strain for recombinant plasmids, was grown in Luria–Bertani (LB) medium at 37°C. Plasmid pBR322 (Boehringer Mannheim) was used as cloning vector.

Detection of lipase activity was performed after growth on CeNAN agar (ADSA-Micro) plates supplemented with tributyrin (1%) or olive oil (1%) and Rhodamine B (0.0002%) [11–12], or by fluorescence emission from cell suspensions [13].

2.2. Nucleic acid manipulation

Plasmid and genomic DNA were purified, manipulated and analysed essentially as described [12]. Restriction nucleases and DNA-modifying enzymes were obtained from Bio-Labs and used according to the manufacturer's specifications. Primer oligonucleotides were purchased at Gibco BRL, and *pfu* polymerase was from Stratagene. DNA

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amplification was usually performed using 29 cycling periods of 30″ at 94°C, 40″ at 42–55°C, and 4′ at 72°C. DNA was sequenced as described [12], homology analysed through BLAST [14], sequence alignments were done using ClustalW (1.74) Multalign programme (http://www2.ebi.ac.uk/clustalw), and signal peptide identification was performed through SignalP V2.0 software [15]. The physico-chemical parameters of the deduced amino acid sequence and presence of defined protein patterns were determined using the Prosite and ProDom databases at ExPASY (http://www.expasy.org).

2.3. Analysis of lipase activity

Activity and zymogram assays were routinely performed using crude cell extracts or concentrated culture supernatants, prepared as previously described [12]. Lipolytic activity of the samples was determined by measuring the release of *para*-nitrophenyl (*p*NP) or 4-methylumbelliferone (MUF) from *p*NP or MUF-derivative substrates, as reported before [12]. One activity unit was defined as the amount of enzyme capable to release 1 μmol of *p*NP or MUF per minute under the reaction conditions used. Zymographic analysis of SDS–PAGE and IEF gels was performed as previously described [13].

2.4. Cloning procedure

Known lipase nucleotide sequences from *B. subtilis* (M74010 and D78508), *B. pumilus* (A34992), *B. licheniformis* (AJ297356), *Bacillus* sp. (AF232707), *B. thermocatenulatus* (X95309), and *B. stearothermophilus* (U78785) were aligned and the regions sharing the highest homology were used to design a set of primers [**FWSUB** (5′-GAT ATT GTG GCT CAT AGT ATG GGC GG) and **BKSUB** (5′-GGC CTC CGC CGT TCA GCC CTT C)] for amplification of an internal lipase-coding DNA fragment from *B. megaterium* 370. Sequencing of the synthesised DNA fragment allowed the design of a new set of specific divergent primers [**BKMEGA** (5′-CCC TCC TAA CGT CAC GAC ATT TTC) and **FWMEGA** (5′-GCT TTC AAA TAG TCA GGT TAA CGC G)], used to perform inverse PCR for the sequencing of the presumed *B. megaterium* 370 lipase-coding gene [16].

The newly amplified DNA fragment showed high sequence identity with *B. subtilis yfi*P gene [17], although several nucleotides were missing at its 3′-end. From the results obtained, a new set of convergent primers corresponding to the 3′- and 5′-ends of the *yfi*P gene sequence [**FWYfiP** (5′-G**TC TAG** AGG GGA ATA AAC GTG AAA AAA G) and **BKYfiP** (5′-G**GC ATG** CAA GAT ATT AAT TTG TAT TGA GG)], was used to perform the final isolation of the complete ORF of *B. megaterium* 370 lipase-coding gene.

The blunt-ended DNA fragment obtained after amplification using primers FWYfiP and BKYfiP was sequenced for confirmation, ligated to *Eco*RV-digested pBR322, and cloned in *E. coli* 5K as described [18] for further characterisation.

2.5. Nucleotide sequence accession number

The DNA sequence of *B. megaterium* 370 (*lip*A_BACME) lipase coding gene was submitted to the EMBL under accession number AJ430831.

3. Results and discussion

3.1. Analysis of B. megaterium 370 lipolytic system

Presence of lipolytic activity in *B. megaterium* 370 was analysed using Rhodamine B-supplemented plates [11], containing tributyrin or olive oil. The strain showed good performance on lipid-based substrate hydrolysis, as confirmed by fluorescence emission from Rhodamine B and appearance of hydrolysis areas around the colonies (not shown).

Activity of concentrated supernatants and cell extracts from *B. megaterium* was analysed on zymograms after SDS–PAGE and IEF separation, using MUF-butyrate as a substrate. A prominent band of ca. 65 kDa and a fainter band of ca. 19 kDa appeared after zymographic analysis of concentrated supernatants from *B. megaterium* 370 (Fig. 1). Study of the supernatants separated on isoelectrofocusing gels confirmed the presence of two activity bands with a p*I* of 5.7 and 9.4, respectively (Fig. 1), indicating that the strain produces more than one extracellular enzyme with activity on MUF-butyrate, being the band of 65 kDa the most abundant at the culture medium.

Zymographic analysis of crude cell extracts from *B. megaterium* 370 separated on SDS–PAGE gels showed the presence of a ca. 58-kDa band with activity on MUF-butyrate (Fig. 1). This band or its processed form was not found at the supernatant, suggesting that the strain would code for an additional non-secreted lipolytic enzyme. IEF zymographic analysis of *B. megaterium* 370 cell extracts confirmed the presence of a single prominent activity band migrating at the acidic area (p*I* 5.5) of the gels (Fig. 1). The p*I* and molecular weight similarity of *B. megaterium* intracellular activity band with those of previously described bacterial carboxylesterases, suggests that this strain could bear a cell-bound esterase similar to PnbA from *B. subtilis* [19], EstA1 from *Bacillus* sp. BP-7 [20], or EstA from *Paenibacillus* sp. BP-23 [12].

3.2. Cloning of a B. megaterium lipase-coding gene

The nucleotide sequences of known *Bacillus* lipases were aligned and the stretches of sequence identity used for the isolation of a *B. megaterium* 370 lipase-coding gene by means of direct and inverse PCR, as described in Section

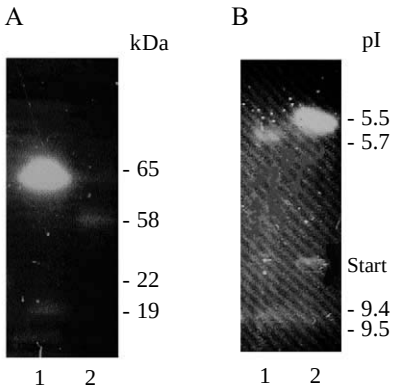

Fig. 1. Zymographic analysis on MUF-butyrate of *B. megaterium* 370 concentrated supernatants (1), and cell extracts (2), analysed by SDS–PAGE (A) and IEF (B) gels. The molecular mass and p*I* of the bands are indicated.

2. A single fragment of ca. 950 bp was obtained and divergently sequenced to get knowledge of the complete ORF. A stretch of 599 nucleotides of an uninterrupted ORF could be known using this procedure, but a small portion of the gene was missing at the 3′-end of the isolated fragment. The known 599-bp sequence of the isolated fragment showed high homology to *B. subtilis yfi*P [17], described later as *lip*B [4]. Given the high homology found to this gene (98%), we used the specific primers (FWYfiP/BKYfiP) to obtain the complete *B. megaterium* lipase-coding gene.

Both control *B. subtilis* and *B. megaterium* 370 produced a common amplification band of ca. 650 bp (not shown) when using the *Yfi*P primers. The amplified 650-bp fragment from *B. megaterium* 370 was isolated and cloned in *E. coli* 5K. Recombinant clones were selected on the basis of tetracycline sensitivity/ampicillin resistance and/or activity on MUF-derivative substrates [13]. As confirmed by Southern hybridisation, the isolated recombinant clone contained a DNA insert from *B. megaterium* 370 (not shown). The cloned lipase, named LipA, was further characterised and its biochemical and molecular properties determined.

3.3. Analysis of the lipase coding sequence

The nucleotide sequence of the cloned lipase was determined and analysed. An open reading frame of 633 nucleotides, coding for a predicted protein of 210 amino acids with a deduced molecular mass of 22 379 Da, and a p*I* of 9.56, was found. As revealed by SignalP programme, a 28-amino acid stretch with the features of the typical *Bacillus* signal peptides [21] was found at the N-terminal region of the protein, indicating its extracellular location. The resulting mature lipase showed a deduced molecular mass of 19 542 Da, and a p*I* of 9.42.

The motif AHS^{78}MG, corresponding to the consensus pentapeptide -A-X_1-S-X_2-G- common in all known *Bacillus* lipases, was found with the first glycine typical residue being replaced by an alanine [1]. Additionally, the cloned lipase contains a His residue at position X_1 like other *Bacillus* lipases, while position X_2 is occupied by a Met, exclusive of subfamily I.4 [6]. The catalytic apparatus of lipases, involving the triad serine, glutamate or aspartate, and histidine [1] was located in the cloned protein, by similarity, at positions 78 (S), 134 (D), and 157 (H).

The deduced amino acid sequence of the cloned enzyme showed the highest homology (98%) to *B. subtilis* LipB [4–17], while lower identity (72%) was found to the remaining *B. subtilis* [3], *B. licheniformis* [6] or *B. pumilus* [5] lipases, all of them grouped into subfamily I.4 [22]. Homology to other Gram-positive or Gram-negative bacterial lipases was much lower (37–15%), indicating that the cloned lipase is a member of a reduced cluster of bacterial serine-esterases grouped in subfamily I.4 of bacterial lipases, exclusive from the mesophilic or moderately thermophilic members of the genus *Bacillus*.

3.4. Properties of the cloned lipase

Recombinant *E. coli* 5K bearing *B. megaterium* 370 LipA was grown on agar plates containing Rhodamine B and supplemented with tributyrin, triolein or olive oil. While clear hydrolysis zones were detected using tributyrin as a substrate, poor activity was found on plates containing triolein or olive oil, the typical substrates for true lipases [1]. Low-fluorescence emission from Rhodamine B was detected from hydrolysis of the substrates assayed, suggesting that the cloned enzyme behaved as an esterase [11].

Cell extracts from the recombinant clone were tested for lipolytic activity on several *p*NP and MUF-derivatives. As shown in Table 1, the cloned enzyme efficiently hydrolysed esters of short-chain fatty acids, while activity decreased as the length of the fatty acid chains increased, a behaviour similar to that described for esterases [1]. Highest activity was found on *p*NP-butyrate, *p*NP-valerate and *p*NP-caproate. When the substrate profile was tested on MUF-derivative substrates, maximum activity was also found

Table 1
Substrate profile of *B. megaterium* Lip A

Substrate	Activity (U/mg protein)
*p*NP-butyrate	0.844
*p*NP-valerate	0.670
*p*NP-caproate	0.620
*p*NP-caprylate	0.547
*p*NP-caprate	0.082
*p*NP-laurate	0.041
*p*NP-palmitate	0.007
*p*NP-stearate	0.004
MUF-butyrate	0.030
MUF-oleate	0.003

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

for esters of short-chain fatty acids (Table 1). According to the proposed model for esterases and lipases, the cloned enzyme showed the profile of an esterase [1].

The effect of pH and temperature on the activity of the cloned enzyme was determined using MUF-butyrate as a substrate. The highest activity was found at 45°C and pH 7.0, exhibiting 87% of the maximum activity at pH 6.0, and 66% residual activity at pH 9.0. The enzyme was stable in a pH range from 4.0 to 12.0 when incubated for 1 h at room temperature. The enzyme remained stable at temperatures ranging from 4°C 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 several agents (1 mM) on the activity of the cloned lipase was determined using *p*NP-laurate, a more stable substrate under the assay conditions used. Hg^{2+} caused almost complete inhibition, incubation in the presence of Ag^{+} led to a significant reduction of activity (52% residual activity), and the rest of ions assayed did not significantly affect the activity of the cloned lipase. From the amino acid modifiers analysed [12], only NBS caused a significant reduction of activity, suggesting that tryptophan could be involved in the functional or structural domains of the cloned enzyme.

The effect of different concentrations of SDS, EDTA, Urea, Phytic acid, Triton X-100® and PMSF was assayed on *p*NP-laurate. High inhibition was caused by Urea, Phytic acid and PMSF, in agreement with previous data on lipolytic enzyme inhibitors [12–20], and with the fact that a serine residue constitutes the active site of the cloned lipase [1]. Triton X-100® caused inhibition at concentrations above 1%, but kept high levels of activity below this concentration. EDTA produced a partial inhibition (40% residual activity) at high concentrations (20 mM), but its effect was not so important at the normal working conditions (1 mM), where the enzyme kept almost 100% of its initial activity. SDS produced an extraordinary activation (eight-fold increase) of the hydrolytic activity at low concentrations (0.05%), but the activity levels shown in the absence of the tensioactive were restored when the SDS concentration was raised to 0.4% values.

SDS–PAGE analysis of crude cell extracts from *E. coli* 5K bearing *B. megaterium* 370 lipase did not show the 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 of ca. 22 kDa that hydrolysed MUF-butyrate, not present in control *E. coli* 5K/pBR322 (Fig. 2). This activity band would correspond to the unprocessed form of the cloned LipA, probably the same as the faint 19-kDa band observed in the zymographic analysis of *B. megaterium* culture supernatants (Fig. 1). This was confirmed by the identical migration of such enzyme at the IEF gels

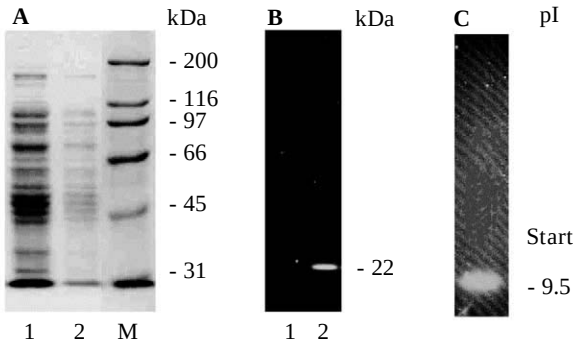

Fig. 2. Coomassie stain (A) and zymograms on MUF-butyrate (B,C) of cell extracts (2) from *B. megaterium* lipase A cloned in *E. coli* 5K, analysed on SDS–PAGE (A,B) or IEF gels (C). Cell extract samples from *E. coli* 5K/pBR322 (1) are shown as a control. The molecular mass and p*I* of the bands are indicated.

from both, cell extracts from the recombinant clone (Fig. 2) and culture supernatants from *B. megaterium* (Fig. 1).

In addition to the cloned LipA, further research is in progress for the isolation and characterisation of the second major secreted lipase and the putative intracellular carboxylesterase of *B. megaterium* 370 to get knowledge of its complete lipolytic system.

Acknowledgements

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Use of methylumbeliferyl-derivative substrates for lipase activity characterization

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Abstract

Lipases and esterases have been recognized as very useful biocatalysts because of their wide-ranging versatility in industrial applications, their stability, low cost, and non-requirement for added cofactors. The physical properties of lipidic substrates, typically water insoluble, have determined a great difficulty in studying lipolytic enzymes. A method for fast and simple detection of lipolytic activity, based on the use of 4-methylumbelliferone (MUF)-derivative substrates was developed. The system has been used for the detection of lipase activity either from microbial colonies, cell culture suspensions, or from proteins separated on SDS-polyacrylamide or isoelectric focusing gels. The use of MUF-derivative substrates has also been extended to the quantitative determination of lipolytic activity from a variety of assays including optimum pH and temperature determination, growth dependency, kinetics or stability studies, or residual activity quantification after treatment with potential inhibitors. The method has shown to be a useful tool for the characterization of a variety of lipases from microbial origin, including those cloned in heterologous hosts.

Keywords: Lipases; Esterases; MUF-butyrate; MUF-oleate; *Bacillus*; *Saccharomyces*; *E. coli*

1. Introduction

Lipases (glycerol ester hydrolases, E.C. 3.1.1.-) are hydrolases acting on the carboxyl ester bonds present in acylglycerols to liberate organic acids and glycerol. Triacylglycerols are mainly uncharged insoluble lipids, although those with short-chain fatty acids are slightly soluble in water. The physical properties of lipids have caused many difficulties in studying the characteristics of lipolytic enzymes [1].

A variety of lipases from microbial origin with different enzymological properties and substrate specificities have been found and characterized [2,3]. The physiological functions of most microbial lipases or esterases are not clear, some of them being probably involved in metabolic pathways to provide carbon sources, in plant cell-wall degradation, in pathogenicity, or in biocide detoxification [4]. However, lipases and esterases have been recognized as very useful biocatalysts because of their great versatility in industrial applications. Practical use of microbial lipases has determined a great interest concerning the improvement of both the producing strains and the biochemical properties of lipolytic enzymes [5,6].

A large number of methods for measuring lipolytic activity have been reported, most of which differ on the systems used for substrate solubilization, and the type of activity marker employed [1,7]. The choice of a method for enzyme activity determination depends on the type of enzymes being studied and the

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information required. Traditionally, lipases have been assayed by radiometric or titrimetric techniques [7]. Radiometric methods are the most sensitive but require the use of radiolabelled and expensive substrates. On the other hand, titrimetric systems suffer from low sensitivity [7]. For experiments involving a large number of assays or non-purified samples, it is convenient to use chromogenic or fluorogenic substrates which can simply and rapidly be tested. Nevertheless, many chromogenic substrates show low specificity and sensitivity, or become spontaneously hydrolyzed under certain conditions, which represents the major disadvantages of using these type of substrates [1].

Therefore, efforts to improve systems for lipase activity detection from purified or non-purified preparations are desirable. The use of lipidic substrates linked to fluorescent compounds provides a sensitive method for detection and quantification of enzyme activity in biological systems [8,9]. Among the fluorogenic substrates, 4-methylumbelliferone-derivatives, commercially available in a wide range of chain-length, have shown to be useful tools for lipase activity determination [9–11]. In this report we describe the use of MUF-derivative substrates for the complete characterization of several lipases well suited for biotechnological applications, some of which have already been cloned and expressed in heterologous hosts [12–15].

2. Experimental

2.1. Strains, plasmids and growth conditions

Bacillus subtilis MB216 [16], *Bacillus* sp. BP-7 [17], and *Paenibacillus* sp. BP-23 [18] were used as gene donors and grown in nutrient broth at 30 °C. *Escherichia coli* 5K [19], *Saccharomyces cerevisiae* strains 13bxV4 (CECT10837, [*trp1*]) and CENPK 113-11A (*trp1-289 his3-Δ1*) [20,21], and *B. subtilis* strains MB216 and BCL1050 [22], were used as recipient hosts for recombinant plasmids. *E. coli* was grown in LB medium at 37 °C. *S. cerevisiae* strains were routinely grown at 30 °C in YPD [21], and occasionally in Difco Minimal Medium [0.17% YNB w/o amino acids +0.5% $NH_4(SO_4)_2$] plus glucose (2%) or olive oil (1%), supplemented in some cases with histidine [23]. Plasmids pBR322, pUC19 [24], YEplac112 [25], and the pUB110-derivative plasmid pRB473 [26] were used as cloning and expression vectors.

2.2. MUF-staining technique

Stock solutions of MUF-butyrate (25 mM) or MUF-oleate (100 mM; Sigma, USA) in ethyleneglycol monomethylether ($C_3H_8O_2$) in the presence of 50 mM phosphate buffer, pH 7.0 were prepared and used for further assays. Lipase producing microbial strains grown on solid medium were detected under UV illumination after covering the plate surface with a 100 μM solution of MUF-butyrate [11]. Detection of lipolytic activity from cell suspensions or cell fractions was performed by transferring a small aliquot (5 μl) of each sample onto filter paper, and addition of 5 μl of 25 mM MUF-butyrate stock solution, followed by UV illumination of the paper. This procedure was also used to select the most convenient working concentration of MUF-substrate for a given sample, or to determine the amount of enzyme required for hydrolyzing a defined MUF-substrate. Detection of lipolytic activity on MUF-butyrate was achieved in less than 1 min, while hydrolysis of MUF-oleate usually required a 15 min incubation at room temperature [11].

2.3. Electrophoresis and zymograms

SDS-PAGE was performed in 10% (w/v) gels, essentially as described by Laemmli [27]. Isoelectric focusing was performed in a Pharmacia Phast System Unit, using gels with a pH range 3.0–9.0. After the run, gels were soaked for 30 min in 2.5% Triton X-100® at room temperature, briefly washed in 50 mM phosphate buffer, pH 7.0, and covered by a solution of 100 μM MUF-butyrate or 200 μM MUF-oleate in the same buffer [11]. Activity bands became visible in a short time after UV illumination. Following zymogram analysis, both SDS-PAGE and IEF gels were subsequently stained with Coomassie Brilliant Blue R®-250, and protein bands visualized.

2.4. Nucleic acid manipulation and cloning procedures

Plasmid and genomic DNA were purified essentially as described [24]. Restriction nucleases

and DNA-modifying enzymes were obtained from Bio-Labs and used according to the manufacturer's specifications. Northern hybridization analysis (not shown) and DNA manipulations were performed as described [24]. Primer oligonucleotides were purchased at Gifco BRL, and *Pfu* polymerase was from Stratagene. DNA was sequenced as described [28], homology analyzed through BLAST [29], and alignments were performed using ClustalW (1.74) Multalign software (http://www2.ebi.ac.uk/clustalw).

Gene libraries from *Paenibacillus* sp. BP-23 and *Bacillus* sp. BP-7 were constructed as described previously [28,13] and the clones coding for esterases EstA and EstA1, respectively, were isolated for further characterization. *B. subtilis* MB216 lipase A gene was isolated by PCR amplification and cloned in several hosts as previously described [14].

2.5. Activity assays

Determination of lipolytic activity was routinely performed from crude cell extracts or concentrated culture media [12]. Activity was determined by measuring the release of *para*-nitrophenol (*p*NP) or 4-methylumbelliferone (MUF) from *p*NP or MUF-derivative substrates. For colorimetric assays, 0.3% stock solutions of *p*NP-derivatives (0.15% for *p*NP-palmitate and *p*NP-stearate) were prepared in isopropanol, and emulsified by sonication [30]. The reaction mixture consisted of 450 μl of a 1:10 dilution of the substrate stock solution in 50 mM phosphate buffer pH 7.0, containing 0.1% Arabic gum and 0.4% Triton X-100®, and 50 μl of cell extract. This mixture was incubated at 37 °C for 15 min, the reaction terminated by addition of 35 μl of 0.1 M Na_2CO_3, and the released *p*NP determined by measuring the absorbance at 410 nm. One unit of activity was defined as the amount of enzyme that released 1 μmol of *p*NP per minute under the assay conditions described.

Fluorometric assays using MUF-derivative substrates were usually performed as time–drive plots in a Hitachi F-2000 spectrofluorimeter (Hitachi, Japan). The fluorescence release caused by hydrolysis of the fluorogenic substrates was measured at $\lambda_{ex} = 323$ nm and $\lambda_{em} = 448$ nm, established as the maximum under our assay conditions. Stock solutions of MUF-derivative substrates (25 or 100 mM) were prepared as described above and emulsified by shaking vigorously [31]. The reaction mixture consisted of 1.5 ml of 50 mM phosphate buffer pH 7.0 containing 0.1% Arabic gum and 0.4% Triton X-100®, to which 6 μl MUF-derivative stock solution and 60 μl cell extract were added. Activity was determined by measuring the increase of fluorescence emission due to the release of MUF caused by hydrolysis, according to previously generated standard plots [12]. One unit of activity was defined as the amount of enzyme required to release 1 μmol of MUF per minute under the conditions described.

Optimum pH was established using 50 mM sodium succinate (pH 4–6), phosphate buffer (pH 6–7.5), Tris–HCl (pH 7–9), and glycine buffer (pH 9–12) in the standard fluorometric assay. To subtract the effect of pH on fluorescence emission, the reactions were terminated by addition of 30 μl 20% HCl so that fluorescence could be measured under the same conditions for all samples. Determination of the optimum temperature or stability was performed by incubating the reaction mixtures or crude cell extracts at different temperatures prior to determination of MUF release. For inhibition studies, assays were performed on *p*NP-laurate and MUF-butyrate in the presence of several metal ions, enzyme modifiers or chemical agents, and the residual activity was measured with respect to that of untreated samples.

3. Results and discussion

3.1. Screening for lipase activity

MUF-derivative substrates were used for the detection and isolation of lipase or esterase-producing microbial strains. This is routinely one the first steps in the study of microbial lipolytic activity. As shown in Fig. 1A, MUF-derivative substrates provide a fast and simple tool for lipolytic activity detection. Lipase producing microbial strains can easily be identified after growth on agar plates, by addition of the chosen MUF-derivative substrate solution onto the plate. A fluorescent signal is visible only for strains bearing lipolytic activity (Fig. 1A).

Once selected the lipase or esterase producing microorganisms on solid media, fast detection of activity in cell suspensions (Fig. 1B) or purified enzyme solutions (Fig. 1C) can be performed by transferring a

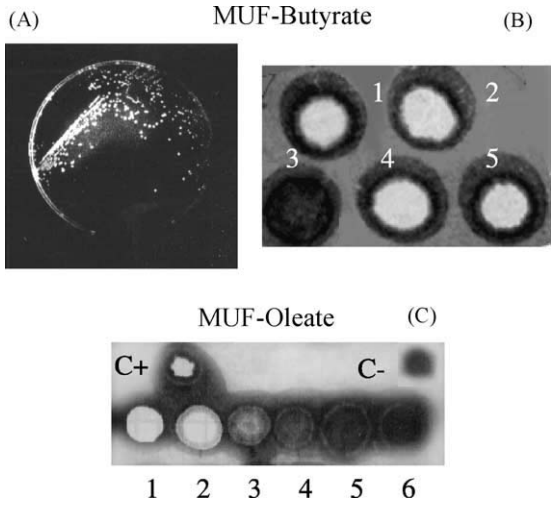

Fig. 1. Activity detection on agar plates and filter paper. (A) Recombinant *E. coli* Xl1 colonies expressing esterase EstA1 from *Bacillus* sp. BP-7. (B) Whole cell suspensions of lipolytic strains *Bacillus* sp. BP-7 (1), *Paenibacillus* sp. BP-23 (2), *Burkholderia cepacia* R6 (4), recombinant *E. coli* 5K expressing EstA from *Paenibacillus* sp. BP-23 (5), and the non-lipolytic strain *E. coli* 5K (3). (C) Several Pancrealipase® dilutions assayed on MUF-oleate; samples 1–6 correspond to 4.9×10^{-6}, 2.45×10^{-6}, 1.22×10^{-6}, 6.1×10^{-7}, 3.0×10^{-7}, and 1.5×10^{-7} enzyme units, respectively. C+ and C− are the control samples.

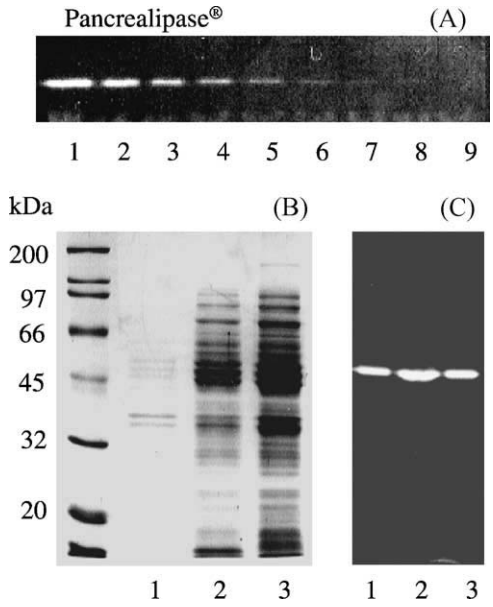

Fig. 2. Zymogram analysis performed on SDS-PAGE gels. (A) Zymogram from an SDS-polyacrylamide gel of different Pancrealipase® dilutions analyzed with MUF-butyrate. Samples (1–9) correspond to 9.9×10^{-6}, 4.9×10^{-6}, 2.45×10^{-6}, 1.22×10^{-6}, 6.1×10^{-7}, 3.0×10^{-7}, 1.5×10^{-7}, 7.5×10^{-8}, and 3.7×10^{-8} enzyme units, respectively. (B) SDS-PAGE of cell fractions from *E. coli* 5K bearing *Paenibacillus* sp. BP-23 esterase A, analyzed for lipolytic activity using MUF-butyrate (right) and subsequently stained with Coomassie Brilliant Blue R®-250 (left) to determine the molecular mass of the active proteins. The samples loaded correspond to peryplasm (1), cytoplasm (2), and membrane-bound proteins (3).

small aliquot of each sample onto filter paper, and addition of a MUF-derivative solution to the samples. UV transillumination of the paper (Fig. 1B) allows the identification of the lipase or esterase producing cells by appearance of a fluorescent signal. The same system can be used as well for the detection of lipolytic activity in different cell fractions, to determine the amount of enzyme required for activity detection, or as a tool to select the most convenient MUF-substrate concentration for a given sample (not shown). Using the described method, 6.1×10^{-7} units of Pancrealipase® could be detected on MUF-butyrate, while 1.2×10^{-6} units of the enzyme were detected on MUF-oleate (Fig. 1C). In all cases, detection of lipolytic activity was achieved in less than 15 min under UV illumination.

3.2. Zymogram analysis

Separation of proteins in polyacrylamide or isoelectric focusing gels is a common analytical technique for protein and enzyme characterization. Conventional staining methods allow the identification of purified lipases, but non-purified enzymes have to be detected through zymograms, usually performed by overlay techniques [32]. MUF-derivative substrates were used for the rapid detection of lipases and esterases separated on SDS-PAGE and IEF gels. Fig. 2A shows a zymogram analysis performed with MUF-butyrate of several Pancrealipase® dilutions separated on a 10% SDS-polyacrylamide gel. In contrast to other zymographic systems [32], the sensitivity range of this technique is extremely high, allowing detection of 1.5×10^{-7} units of Pancrealipase® on MUF-butyrate in less than 15 min. The short time required for activity detection on gels greatly contributes to prevent protein diffusion, thus allowing a most accurate determination of the protein molecular weight. Nevertheless, the degree

of sensitivity of the system is a function of the properties of the enzyme analyzed and the substrate used. According to this, higher amounts of enzyme were needed (2.5×10^{-6} units of Pancrealipase®) when MUF-oleate was used as a substrate in the zymogram (not shown).

An important advantage of the zymographic technique described is that after activity detection, the same gels can subsequently be stained with a conventional dye in order to determine the molecular mass of the active proteins (Fig. 2B and C). This fact and the lack of protein diffusion due to the short reaction time allow a great accuracy in the determination of the molecular weight of a given enzyme, not provided by the conventional overlay systems or by analysis of proteins and activity in separate gels. Fig. 2B and C show the same gel containing different cell fractions of lipolytic strain *Paenibacillus* sp. BP-23, analyzed as a zymogram (Fig. 2C), and subsequently stained with Coomassie Brilliant Blue R®-250 (Fig. 2B). The active bands detected by the zymogram are not visible at the Coomassie Blue stained gel due to their low protein contents. Zymographic analysis with MUF-derivatives was also used for the determination of the isoelectric point of several lipolytic enzymes (not shown). Like in the case of PAGE, IEF gels can subsequently be stained by conventional dyes, and the pI of the active proteins established with great accuracy. Traditional zymographic analysis of lipases are less sensitive, time consuming and more expensive procedures. For this reason, the higher effectiveness of the method described makes it a very valuable tool for straightforward detection of lipolytic enzymes even from non-purified or non-concentrated samples [11].

3.3. Characterization of lipases using MUF-derivative substrates

Biochemical characterization of enzymes requires the use of systems that allow accurate quantification of activity. We extended the use of MUF-derivative substrates to the determination of lipolytic activity in samples from different origin and under a variety of conditions.

Growth dependency of enzyme production by microbial strains or by recombinant microorganisms bearing lipase genes was evaluated by spectrofluorometry assays, as described in the experimental section. Fig. 3 shows the production of lipolytic activity along growth of *Paenibacillus* sp. BP-23 strain (Fig. 3A), and the production of lipase A in parental strains and recombinant clones of *S. cerevisiae* bearing *B. subtilis lip*A gene (Fig. 3B). Fig. 3A shows that there is a growth-dependent expression of lipases in strain *Paenibacillus* sp. BP-23, as the highest amount of lipolytic activity appeared after 24 h incubation. On the contrary, expression of *B. subtilis* lipase A in recombinant *S. cerevisiae* strains is mainly constitutive, with a maximum peak of production after 24 h growth. Activity was found at the cell extract fractions of these clones from the beginning of the exponential phase for both MUF-butyrate (Fig. 3B) and MUF-oleate (Fig. 3C) substrates. These results are in agreement with the use of a constitutive yeast promoter in the recombinant constructions [14]. As expected, parental yeast strains lacking lipase A, used as control samples, showed negligible lipolytic activity.

Lipases exhibit different kinetic behaviors depending on the properties and concentration of the substrate they hydrolyze. An interfacial activation of true lipases occurs at high substrate concentration or when long chain-length substrates are used. On the contrary, esterases and other lipolytic enzymes display a Michaelis–Menten behaviour, without interfacial activation [1,3]. Therefore, we used the MUF-derivative substrate-based spectrofluorometric method to assay the substrate specificity and kinetic parameters of lipases. Table 1 shows the results obtained for *Paenibacillus* sp. BP-23 EstA, *Bacillus* sp. BP-7 EstA1, and *B. subtilis* LipA, assayed from crude cell extracts of the corresponding clones in *E. coli* 5K. In all cases, activity was higher on MUF-butyrate, indicating a preference of these enzymes for short chain-length substrates. A significant decrease of activity was observed as the length of the fatty acid chains increased, a behavior similar to that described for true esterases [1]. The kinetic parameters of the cloned enzymes were analyzed on MUF-butyrate and MUF-oleate. For both substrates, a standard Michaelis–Menten plot was obtained (not shown), with the apparent constants shown in Table 1. According to the proposed model for esterases and true lipases, the three enzymes displayed the typical properties of esterases, showing no interfacial activation by substrate concentration. The results obtained are not surprising for *Paenibacillus* sp. BP-23

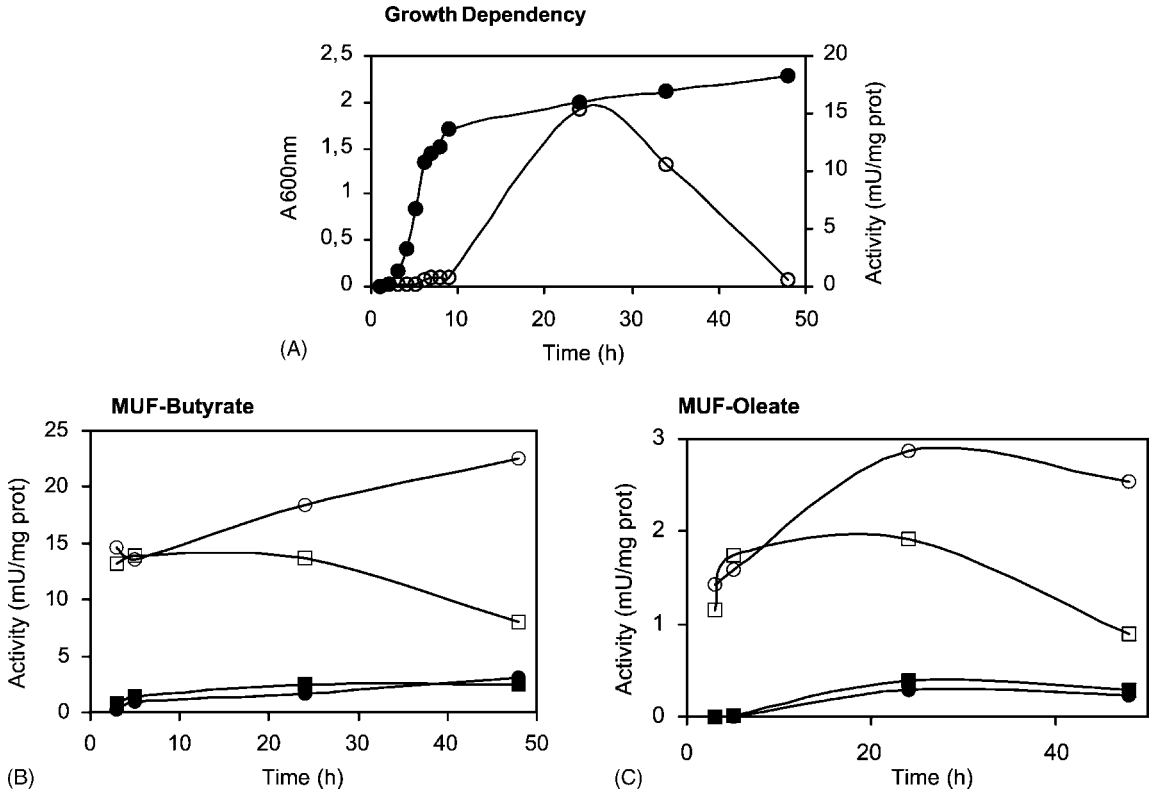

Fig. 3. Use of MUF-derivative substrates for lipase activity determination. Growth dependency of lipase activity production in *Paenibacillus* sp. BP-23; (●) absorbance at 600 nm, (○) secreted activity, measured with MUF-butyrate (A). Cell extract lipase activity found on MUF-butyrate (B) or MUF-oleate (C) for *S. cerevisiae* strains 13bxV4 (squares) and CENPK113-11A (circles) transformed with plasmid YE[pACT1-ssInv-LipA-T] (empty symbols) or YEplac112 (control samples, solid symbols), grown in YPD medium.

EstA or *Bacillus* sp. BP-7 EstA1, previously described as esterases [12,13]. Although *B. subtilis* LipA has for a long time been considered a true lipase, our results agree with recent reports indicating that no interfacial activation is required for activity, as the enzyme lacks a structural α-helix fold that acts to prevent true lipases from displaying activity in the absence of an interface [4,33,34].

A number of enzyme activity inhibitors were also studied by fluorometric techniques using MUF-derivatives. Fig. 4B shows an example of enzyme inhibition caused by phytic acid on Pancrealipase®. Increasing concentrations of phytic acid were added to the reaction mixture, and residual activity was measured using MUF-butyrate (Fig. 4B) or MUF-oleate (not shown). A concentration of 0.31% phytic acid

Table 1
Specific activity and K_m values of tested lipases for different MUF-derivative substrates

	Paenibacillus sp. BP-23 EstA		*Bacillus* sp. BP-7 EstA1		*Bacillus subtilis* LipA	
	Units (mg)	K_m (mM)	Units (mg)	K_m (mM)	Units (mg)	K_m (mM)
MUF-butyrate	9.5	0.0155	0.28	0.026	0.13	0.031
MUF-oleate	1.2×10^{-4}	0.029	3×10^{-3}	0.055	0.02	0.044

Activity values are the mean of three independent assays.

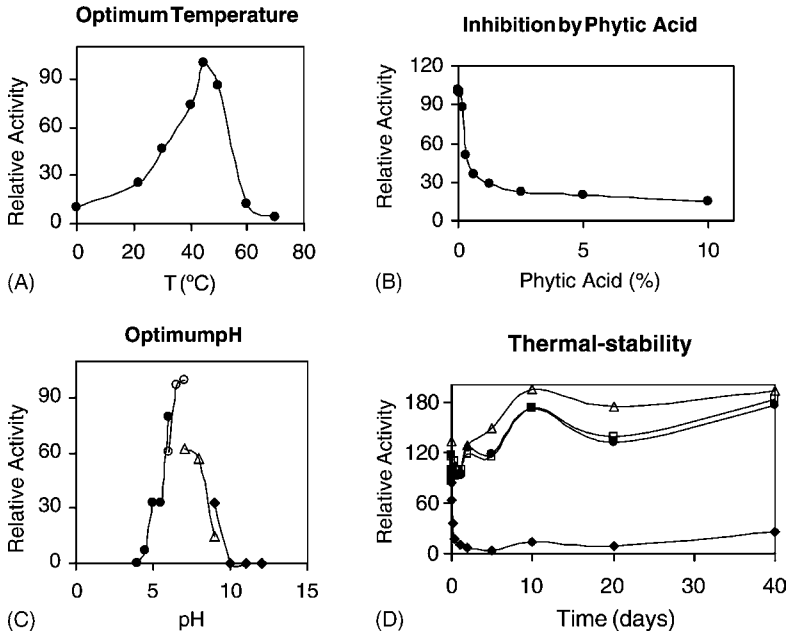

Fig. 4. Characterization of lipases using MUF-derivative substrates. (A) Optimum temperature determination for *E. coli* 5K cell extracts bearing EstA1 from *Bacillus* sp. BP-7, using MUF-butyrate. (B) Inhibition of Pancrealipase® activity caused by phytic acid, measured with MUF-butyrate. (C) Optimum pH determination for *E. coli* 5K cell extracts bearing EstA1 from *Bacillus* sp. BP-7, using MUF-butyrate. The reaction was performed at different pH in the presence of succinate (●), phosphate (○), Tris–HCl (△), and glycine (◆) buffers. (D) Thermal stability of *Bacillus megaterium* lipase A cloned in *E. coli* 5K, measured using MUF-butyrate. Temperatures assayed were: 4 °C (△), 20 °C (□), 30 °C (●), and 45 °C (◆). The highest relative activity value for each parameter analysed was set at 100.

caused a 50% reduction in Pancrealipase® activity on MUF-butyrate. However, we must point out that when inhibition assays are performed using MUF-derivative substrates, quenching of fluorescence [35], potentially caused by the added substances or their solvents, must be taken into account, and accurate standard plots must be performed for each compound as well as for the solvents used. In all our determinations, standard plots for both the compound and the solvent were constructed and used to calculate the quenching effect.

Determination of optimum pH and temperature for lipase activity was also performed using MUF-derivative substrates. However, both parameters cause important effects on fluorescence emission [35]. For this reason, a simple modification of the standard protocol was established, based on stopping the reaction with HCl and bringing all samples to the same pH and temperature conditions before measuring the fluorescence emission. Fig. 4 shows the determination of optimum temperature (Fig. 4A) and pH (Fig. 4C) for *Bacillus* sp. BP-7 EstA1 esterase cloned in *E. coli* 5K, performed using MUF-butyrate. As shown in the plots, maximum activity of the enzyme was achieved at pH 7.0 and 45 °C. The system described was also successfully used for measuring the thermal (Fig. 4D) or pH stability of lipases and to study the need for cofactors of these enzymes (not shown). Lipase A from *B. megaterium* [15] displayed good thermal stability in the temperature range from 0 to 30 °C, being fully active for at least 40 days (Fig. 4D). When incubated at 45 °C, a rapid decrease of activity was detected, reaching 50% of the initial activity after 2.5 h incubation. All the activity determinations were performed in triplicate, and control plots were obtained for each sample. The reliability of the method was studied using *para*-nitrophenyl-derivatives as alternative substrates. The results obtained following the protocol described in the experimental section [12] (not shown) confirmed the reproducibility of the MUF-based system.

The results obtained indicate that detection and determination of lipase activity using MUF-derivative substrates provides a fast, sensitive and accurate system to gain information about the biochemical and molecular properties of lipases, allowing thus to take rapid and valuable decisions about the potential biotechnological applications of the lipases studied.

4. Conclusions

The fluorescence-based lipase activity assay described here provides an excellent tool to identify lipolytic microorganisms and to study the biochemical properties of lipolytic enzymes. The remarkable sensitivity, speed and simplicity of this standarized system may contribute to eliminate the difficulties posed by most of the traditional methods for lipase assay reported in the literature, and may help in the search for new biotechnologically useful enzymes.

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

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ABSTRACT

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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 *lip*A 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.*

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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-methyl-umbelliferone (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 (*lip*A_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 S^{78}, D^{134} and H^{157}. 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

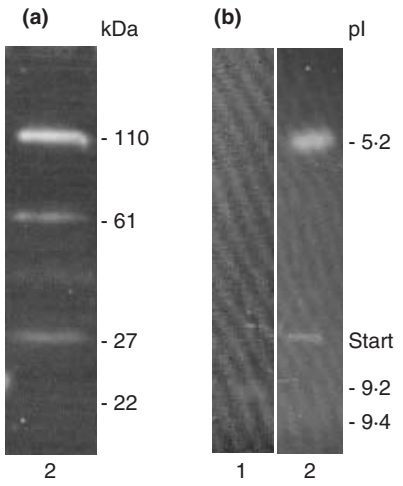

Fig. 1 SDS-PAGE and IEF zymograms on MUF-butyrate of *Bacillus* sp. BP-6 lipases. (a) Zymogram analysis of cell extracts from *Bacillus* sp. BP-6 (2) separated by SDS-PAGE. (b) IEF zymogram of *Bacillus* sp. BP-6 concentrated supernatants (1) and cell extracts (2). The molecular mass and *p*I of the 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 *p*NP 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)
*p*NP-butyrate	1·410
*p*NP-valerate	1·040
*p*NP-caproate	0·810
*p*NP-caprylate	0·320
*p*NP-caprate	0·080
*p*NP-laurate	0·026
*p*NP-palmitate	0·011
*p*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). Hg^{2+} caused high inhibition, while Ag^{+} led to a significant reduction of activity. Partial inhibition was caused by 10 mM concentrations of Fe^{3+}, Cu^{2+}, Pb^{2+} and Zn^{2+}, 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 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 *p*I (9·4) of these activity bands correspond to those deduced for the unprocessed form of LipA.

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

	Activity (%)	
Agent	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_4Cl	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.

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 form 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*

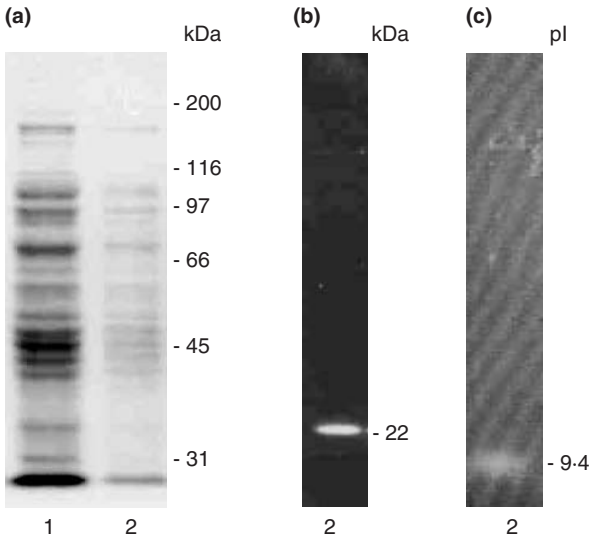

Fig. 2 Analysis of cloned *Bacillus* sp. BP-6 LipA. Coomassie stain (a) and zymograms on MUF-butyrate (b, c) of cell extracts from *E. coli* 5K/pBR322 (1) and recombinant clone *E. coli* 5K/pBP6 (2), analysed by SDS-PAGE (b) and IEF (c). The molecular mass and *p*I of the bands are indicated

(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 *p*I 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 ubiquous 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 (Pohlenz *et al.* 1992), or in the conversion of low-cost fats into added value products (Schmidt-Dannert 1999).

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Activation and inhibition of *Candida rugosa* and *Bacillus*-related lipases by saturated fatty acids, evaluated by a new colorimetric microassay

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Abstract

Research on lipase inhibitors could help in the therapy of diseases caused by lipase-producing microorganisms and in the design of novel lipase substrate specificities for biotechnology. Here we report a fast and sensitive colorimetric microassay that is low-cost and suitable for high-throughput experiments for the evaluation of lipase activity and inhibition. Comparison of *Candida rugosa* activity and inhibition with previous HPLC results validated the method, and revealed the importance of the reaction mixture composition. The assay was used to evaluate the effect of saturated fatty acids on *Bacillus*-related lipases. Cell-bound esterases were strongly inhibited by fatty acids, suggesting a negative feedback regulation by product, and a role of these enzymes in cell membrane turnover. *Bacillus subtilis* LipA was moderately activated by low concentrations of fatty acids and was inhibited at greater concentrations. LipB-like esterases were highly activated by myristic and lauric acids and were only slightly inhibited by high capric acid concentrations. Such an activation, reported here for the first time in bacterial lipases, seems to be part of a regulatory system evolved to ensure a high use of carbon sources, and could be related to the successful adaptation of *Bacillus* strains to nutrient-rich environments with strong microbial competition.

Keywords: Lipase; Colorimetric; Saturated fatty acid; *Bacillus*

1. Introduction

Lipases and esterases are glycerol ester hydrolases (E.C. 3.1.1.-) acting on acylglycerols to liberate fatty acids and glycerol. Several lipases produced by microbial pathogens play an important role in infective diseases. Indeed, *Propionibacterium acnes* lipase and its inhibition by anti-acne compounds have been widely studied because the fatty acids produced by *P. acnes* lipase activity on sebaceous triglycerides induce severe inflammation [1]. Moreover, *Helicobacter pylori* lipase activity can weaken the barrier properties of mucus by hydrolyzing endogenous lipids [2–4], and it is inhibited by sucralfate and other antiulcer drugs [5,6]. Therefore, research on new lipase inhibitors for the therapy of these diseases has generated a great interest.

On the other hand, lipolytic enzymes are currently attracting an enormous attention because of their biotechnological potential [7]. Inhibition studies on lipases could contribute to better understand the mechanism of action of these enzymes in order to design novel substrate specificities for catalyzing specific reactions useful for industrial applications [8] and new inhibitory compounds of either pharmacological or biotechnological interest.

A large number of methods for measuring lipolytic activity and inhibition have been reported [9,10]. These methods differ in the process used for substrate solubilization, in the activity marker employed, and in the detection system. Most of them are not suitable for non-purified samples or for a large number of assays because they are expensive or time-consuming. Efforts to automation and miniaturization are desirable, and chromogenic and fluorimetric assays are by far the simplest, the most reliable, and the easiest for large-scale experiments [10].

Abbreviations: Crl, *Candida rugosa* lipase; $A_{405\ nm}$, absorbance at λ=405 nm; IC, inhibitory concentration; *p*-NP, *p*-nitrophenol; *p*-NPL, *p*-nitrophenyl laurate; β-NL, β-naphthyl laurate; *Pb.*, *Paenibacillus*; *B.*, *Bacillus*

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Here we report a new colorimetric microassay for the evaluation of lipolytic activity and the effect of potential inhibitors based on a previously described colorimetric method [11]. *Candida rugosa* lipase (Crl), a well-known enzyme widely used in biotechnology and commercially available [7,35], was used to validate the assay. Inhibition of *C. rugosa* enzymes is also interesting because some strains, refractory to antifungal therapy, are relevant in veterinary mycology and in emerging pathogenesis in immunocompromised patients [12].

On the other hand, *Bacillus* lipases have attracted a great interest due to their biotechnological potential [13]. The physiological role of these lipases is still unclear, but they may be involved in providing carbon sources, in pathogenicity, or in detoxification of biocides [14]. Among them, *Bacillus subtilis* LipA and LipB are the best known enzymes. LipA-related lipases are more variable while LipB-like esterases display over 98% identity even in very unrelated strains [15]. Structurally, LipA and LipB share a conserved core, having in the protein-surface 43 out of the 45 different residues, but without affecting the free access of the solvent to the active site [16]. LipA transcription is constitutive and independent from glucose, tributyrin, *n*-hexadecane, NaCl or ethanol, but is repressed by amino acids. This lipase is permanently inactivated when it is secreted at pH 5. On the contrary, LipB transcription only takes place in rich media, being independent from glucose, NaCl, ethanol or amino acids, although it is activated by tributyrin and *n*-hexadecane. The enzyme is not inactivated when it is secreted at pH 5 [16,17]. The differences mentioned suggest different physiological functions of these lipases; however, further studies are necessary to know their regulation.

The effect of saturated fatty acids on *Bacillus* lipases is yet unknown despite that they are products of lipase activity and that they are well-known competitive inhibitors of the hydrolysis and synthesis reactions catalyzed by lipases [18,19]. Moreover, fatty acids are known to be involved in the regulation of microbial growth [20], lipase production [1] and in the expression of virulence factors [21]. Thus, the new colorimetric assay was used to evaluate their effect on five *Bacillus*-related lipolytic enzymes: *Paenibacillus* sp. BP-23 EstA [11] and *Bacillus* sp. BP-7 EstA1 [22], two cell-bound type B esterases similar to *B. subtilis* PnbA [23]; and three secreted enzymes displaying 50% similarity to the central region of *P. acnes* lipase, the lipase LipA from *B. subtilis* [24], and the esterases LipA from *B. megaterium* [25] and LipA from *Bacillus* sp. BP-6 [26].

2. Materials and methods

2.1. Reagents

Methanol, acetonitrile, and water for HPLC were purchased from Lab-Scan (Dublin, Ireland). Trifluoroacetic acid and ethyl acetate were from Aldrich (Milwaukee, WI, USA). *p*-Nitrophenol (*p*-NP), *p*-nitrophenyl laurate (*p*-NPL), β-naphthyl laurate (β-NL) and 2-propanol were obtained from Fluka (Buchs, SG, Switzerland). *C. rugosa* lipase (Crl, cat. no. L-1754) and the other reagents were from Sigma (St. Louis, MO, USA). All reagents were used without further purification.

2.2. Preparation of Bacillus-related lipases

Crude cell extracts containing (*Paeni*)*Bacillus* lipases were prepared essentially as described [11]. Overnight cultures of the corresponding *Escherichia coli* recombinant clones were centrifuged for 10 min at 4000 × *g*. The pellets were suspended in 50 mM phosphate buffer (pH 7 at 22 °C), sonicated, and cleared by centrifugation for 10 min at 10 000 × *g*. The supernatants, considered as the lipase-containing cytoplasm fractions, were recovered for lipase inhibition assays.

2.3. Evaluation of lipase activity by a new colorimetric microassay

The non-colored substrate *p*-NPL was dissolved at 20 mM in 2-propanol by sonication for 3 min. A 1:10 (v/v) dilution in phosphate-Triton X-100 buffer was prepared with gentle agitation until an optically clear and stable emulsion was achieved. Fifty-microliter doses of this mixture were dispensed into a 96-well microtiter plate and preincubated for 15 min at 37 °C. Fifty microliters of each enzyme solution (0–5 mg ml^{-1}), prepared in 50 mM phosphate buffer (pH 7 at 22 °C) and previously preincubated for 15 min at 37 °C, were added to the substrate mixture to obtain final 100-μl reaction mixtures (1 mM *p*-NPL, 5% 2-propanol, 0.6% Triton X-100, 50 mM phosphate buffer (pH 7 at 22 °C), and 0–2.5 mg ml^{-1} of enzyme), which were incubated for 15 additional minutes at 37 °C. The absorbance at $\lambda = 405$ nm ($A_{405\ nm}$) of each well was measured in a microtiter plate reader (BIO-RAD Model 3550) to detect the release of *p*-NP, the yellow-colored product of the reaction. Proper blanks, corresponding to the absorbance of the reaction mixture other than that produced by *p*-NP, were determined and subtracted from the total absorbance. All assays were performed within the linear range of absorbance of the *p*-NP calibration curve, which was obtained under strict assay conditions to avoid the strong absorbance changes of *p*-NP at different pH values [27]. The highest protein concentrations within the linear range of activity vs. protein-concentration curves were chosen for further experiments and to estimate the enzymatic activity with respect to the *p*-NP calibration curve. One unit of activity (U) was defined as the enzymatic activity that released 1 μmol of *p*-NP per hour under the assay conditions.

2.4. Evaluation of the effect of saturated fatty acids on lipase activity

Capric, lauric and myristic acids were dissolved in 2-propanol by sonication for 3 min. Assays were performed in

microtiter plates as described above and the $A_{405\ nm}$ was measured from each reaction mixture containing the following final concentrations: fatty acid (from 0 to their maximum emulsion in the mixture), 1 mM *p*-NPL, 5% 2-propanol, 0.6% Triton X-100, 50 mM phosphate buffer (pH 7 at 22 °C), and enzyme (10^{-2} mg ml^{-1} of Crl and $0.8–8.5 \times 10^{-2}$ mg ml^{-1} of *Bacillus*-related lipases). Crl inhibition assays were also performed in a reaction mixture using, instead of phosphate buffer, 50 mM Tris–HCl buffer (pH 7.4 at 22 °C) containing 3.5 mM NaCl and 1.5 mM $CaCl_2$.

Lipase inhibition or activation was calculated from the residual activity detected in the presence of the compound under assay with respect to that of untreated samples, subtracting the absorbance of the reaction mixtures with proper blanks. The concentrations yielding a lipase inhibition of 16% (IC_{16}) and 50% (IC_{50}) were calculated from the inhibition vs. fatty acid concentration curves by regression analysis. All experiments were done at least in duplicate. The intra- and the inter-assay coefficients of variation were calculated based on the analysis of Crl inhibition by 15 mM capric acid.

2.5. Evaluation of lipase activity by HPLC

Lipase activity assays were performed as described [28] with the following modifications: 200-μl reaction mixtures containing 0.46 mM β-NL, 5% acetone, 0.6% Triton X-100, 50 mM phosphate buffer (pH 7 at 22 °C) and $0–5 \times 10^{-2}$ mg ml^{-1} of Crl, were incubated for 15 min at 37 °C under gentle mixing. Then, β-naphthol was extracted with 200 μl of ethyl acetate, and 75 μl of the organic phase was withdrawn, evaporated at room temperature under a nitrogen stream and redissolved in 150 μl methanol. Aliquots of 50 μl were analyzed at room temperature by HPLC as described [28], using a C-18 reversed-phase column (4.6 × 250 mm; 5 μm particle size, 90 Å pore size; Beckman) equilibrated at a flow rate of 1 ml min^{-1}, with a mobile phase consisting of 40% (v/v) acetonitrile in water, containing 0.1% trifluoroacetic acid. The eluate was monitored at a wavelength of 230 nm with a sensitivity of 0.8 A.U.F.S. using a recorder chart speed of 0.5 cm min^{-1}. The chromatographic system consisted of a precision pump (Waters, model 515) and a variable wavelength monitor (Waters, model 2487). The area under the chromatographic peak was measured using the Millennium 32 chromatography manager 4.0 software package for Windows®. β-NL unspecific hydrolysis was eliminated with proper blanks, and the enzymatic activity determined by estimating the amount of β-N released from β-NL hydrolysis under the conditions described.

2.6. Statistics

Regression analyses were done using the Sigma-Plot 8.0 program (SPSS, Chicago, IL, USA).

3. Results

3.1. Evaluation of lipase activity by a new colorimetric microassay

A new colorimetric assay was developed, which features a clear and stable substrate emulsion. Under the assay conditions, the $A_{405\ nm}$ of the *p*-NP calibration curve was linear up to 1.8, corresponding to a *p*-NP concentration of 0.5 mM. Activity vs. protein-concentration curves were determined for Crl (Fig. 1A) and *Bacillus*-related lipases (data not shown). The highest protein concentrations within the linear range of these curves were: 10^{-2} mg ml^{-1} (Crl) and $0.8–8.5 \times 10^{-2}$ mg ml^{-1} (*Bacillus*-related lipases). The enzymatic activities calculated from these concentrations were: 138, 173, 19.0, 30.6, 14.4 and 14.4 U mg^{-1} of protein for Crl, EstA, EstA1, *B. megaterium* LipA and *Bacillus* sp. BP-6 LipA, respectively. Activity vs. *p*-NPL-concentration plots were also obtained showing that all enzymes displayed a Michaelis–Menten behaviour (Crl: Fig. 1C; (*Paeni*)*Bacillus* lipases: data not shown).

3.2. Comparison of Crl activity and inhibition between the colorimetric and HPLC methods

As is shown in Fig. 1, both methods were equivalent when Crl activity was assayed. In fact, activity vs. Crl-concentration curves had the same linear range (up to $1.2–1.5 \times 10^{-2}$ mg ml^{-1}) for the colorimetric assay (Fig. 1A) as for the HPLC assay (Fig. 1B). In both cases, the enzymatic activity estimated was similar (138.4 and 113.5 U mg^{-1} of Crl, respectively). Moreover, activity vs. substrate-concentration plots showed an equivalent kinetic behaviour when measured either through the colorimetric (Fig. 1C) or the HPLC (Fig. 1D) assays.

In agreement with the results obtained previously by HPLC [28] (Table 1C), when the effect of saturated fatty acids on Crl was analyzed using the colorimetric assay (Table 1A; Fig. 2), a similar low inhibitory effect was observed for the three fatty acids assayed. Small differences in the inhibition were found between the two methods, but they drastically decreased, mainly at low fatty acid concentrations, when the colorimetric assay was performed using reaction mixture conditions similar to those of the HPLC method (Table 1B). The intra- and inter-assay coefficients of variation of the colorimetric assay were about 3% and 4%, respectively.

3.3. Effect of saturated fatty acids ($C_{10}–C_{14}$) on Bacillus-related lipases

Table 2 and Fig. 2 show the effect of capric, lauric and myristic acids on (*Paeni*)*Bacillus* lipolytic enzymes, evaluated using the colorimetric assay. *Paenibacillus* sp. BP-23 EstA and *Bacillus* sp. BP-7 EstA1 displayed almost the same profile as Crl, showing a similar inhibition for all fatty

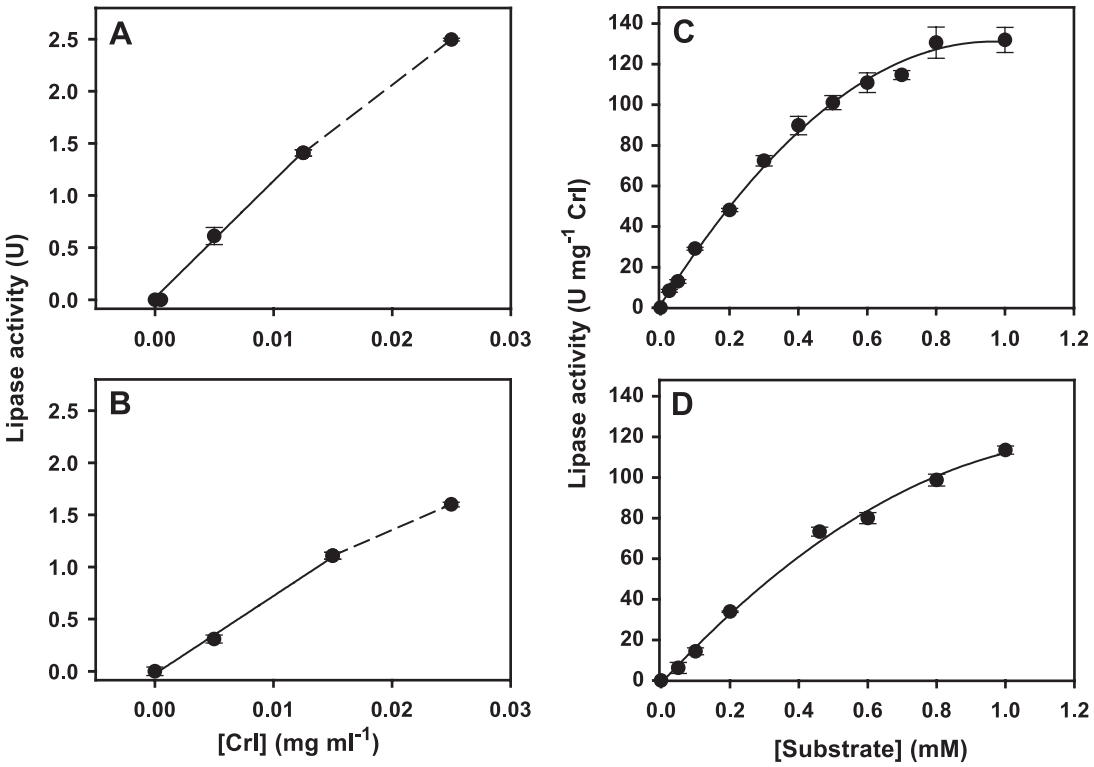

Fig. 1. Comparison of *C. rugosa* activity evaluated by colorimetric assay and by HPLC. Colorimetric microassay (A, C); HPLC (B, D). The continuous line shows the linear range of the activity vs. CrI concentration curves (A, B). Activity vs. *p*-nitrophenyl laurate concentration plots are also displayed (C, D).

acids assayed. Myristic acid produced the lowest inhibition, mainly at high concentrations. Among the lipases tested, EstA1 revealed the strongest inhibition by fatty acids: IC_{50} = 3.8–8.5 mM; 11% relative activity at 20 mM capric acid (Table 2, Fig. 2).

B. megaterium LipA and *Bacillus* sp. BP-6 LipA showed a completely different behaviour, being highly activated by saturated fatty acids. Myristic acid, the strongest activator compound, showed relative activities above 225–240% from 2.5 to 15 mM, with a maximum of 285% at 5 mM. Lauric acid displayed a high activation plateau (relative activity 220–230%) at 2.5–10 mM, whereas the activation decreased with higher concentrations (125–130% relative activity at 20 mM). Capric acid caused the lowest activation of these enzymes (155–160% relative activity at 2.5–5 mM), with the activation decreasing even to 90% (10% inhibition) at 25 mM fatty acid concentration (Fig. 2).

B. subtilis LipA reported an intermediate behaviour between cell-bound and secreted esterases. All fatty acids produced a moderate activation (140–150% relative activity) at 2.5–5 mM. The activation decreased at higher concentrations to almost disappearance at 15 mM. Above 15 mM, an increasing inhibition was found (nearly 50% inhibition at 25 mM capric acid) (Table 2, Fig. 2).

Table 1
C. rugosa lipase inhibition by fatty acids evaluated by several methods

Fatty acid	S_{max} (mM)	A		B		C	
		IC_{16} (mM)	IC_{50} (mM)	IC_{16} (mM)	IC_{50} (mM)	IC_{16} (mM)	IC_{50} (mM)
Capric acid (C10)	25	4.2	19.4	4.7	14.3	4.5	11
Lauric acid (C12)	20	4.1	14.7	1.3	14.5	1.4	10
Myristic acid (C14)	15	4	~ S_{max}	1.1	13.6	2.6	10

A: colorimetric assay. Reaction mixture: 1 mM *p*-NPL, 5% 2-propanol, 0.6% Triton X-100, 50 mM phosphate buffer (pH 7 at 22 °C) and 10^{-2} mg ml^{-1} of CrI.

B: colorimetric assay. Reaction mixture: 1 mM *p*-NPL, 5% 2-propanol, 0.6% Triton X-100, 3.5 mM NaCl, 1.5 mM $CaCl_2$, 50 mM Tris–HCl buffer (pH 7.4 at 22 °C) and 10^{-2} mg ml^{-1} of CrI.

C: Grippa et al. HPLC values [28]. Reaction mixture: 0.46 mM β-NL, 5% acetone, 1 mM sodium taurocholate, 3.5 mM NaCl, 1.5 mM $CaCl_2$, 50 mM Tris–HCl buffer (pH 7.4 at 22 °C) and 10^{-2} mg ml^{-1} of CrI.

S_{max}: highest final concentrations of fatty acid achieved in well-emulsified reaction mixtures.

4. Discussion

A previously described colorimetric assay for the evaluation of lipase activity and inhibition [11] was adapted and simplified in order to obtain a fast, simple, accurate and more sensitive colorimetric microassay that is low-cost and suitable for working with large numbers of samples using either purified or non-purified lipases.

Methods of measuring lipase activity using *p*-NP-esters should be used with care as the carbonyl function of these esters is electronically activated, which makes them liable

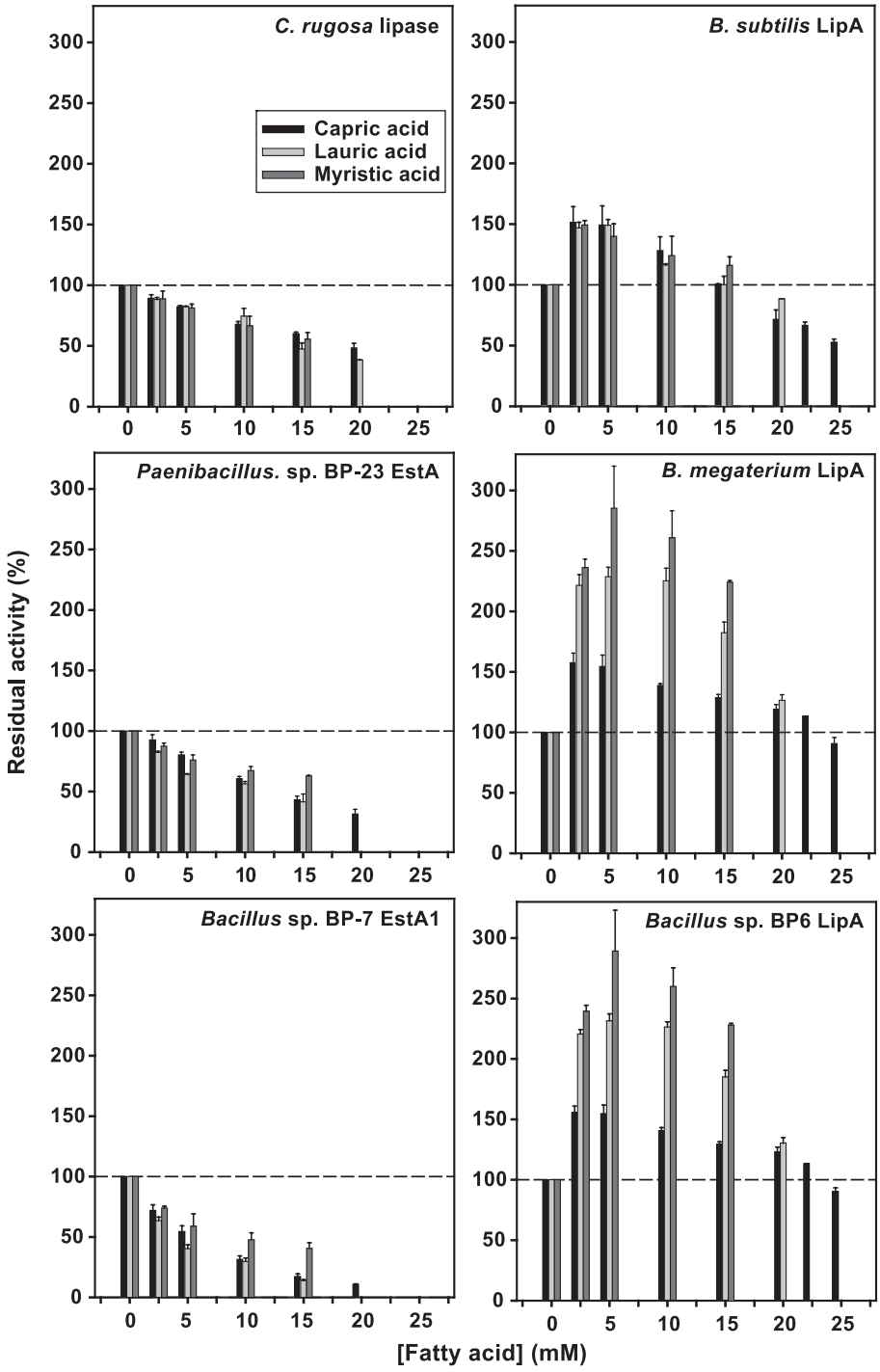

Fig. 2. Effect of capric, lauric and myristic acids on *C. rugosa* and *Bacillus*-related lipases. Lipase activity in absence of any fatty acid (100% residual activity) is indicated by dotted lines. Greater and lower residual activities mean activation and inhibition by fatty acids, respectively.

to undergo nonenzymatic hydrolysis under drastic pH conditions [9,10]. However, this disadvantage no longer applies when long acyl-chain *p*-NP-esters and inert micellar detergents are used [9]. For this reason, we chose Triton X-100 as nonionic detergent, and *p*-NPL as substrate, which is stable and shows an intermediate behavior between short and long, low-soluble *p*-NP-esters. In fact, *p*-NPL micellized with Triton X-100 is a good physicochemical system widely used for lipolytic assays because the surface of the micelles is reproducible and easy to handle, and *p*-NPL spontaneous hydrolysis is negligible during 8 h [29]. Obtaining a clear and stable substrate emulsion made it possible to perform the assays using the same initial reaction mixture for all samples, without any mixture-clearing centrifugation step. This allowed us to use multichannel pipettes and microtiter plates to automate and

Table 2
Inhibition of *Bacillus*-related lipases by fatty acids

Fatty acid	S_{max} (mM)	*Paenibacillus* sp. BP-23 EstA		*Bacillus* sp. BP-7 EstA1		*Bacillus subtilis* LipA		*Bacillus megaterium* LipA		*Bacillus* sp. BP-6 LipA	
		IC_{16} (mM)	IC_{50} (mM)	IC_{16} (mM)	IC_{50} (mM)	IC_{16} (mM)	IC_{50} (mM)	IC_{16} (mM)	IC_{50} (mM)	IC_{16} (mM)	IC_{50} (mM)
Capric acid (C10)	25	4.3	12.8	1.3	5.6	18.9	~ S_{max}	>>S_{max}		>>S_{max}	
Lauric acid (C12)	20	2.0	12.6	0.9	3.8	>S_{max}		>>S_{max}		>>S_{max}	
Myristic acid (C14)	15	3.0	~ S_{max}	1.2	8.5	>S_{max}		>>S_{max}		>>S_{max}	

S_{max}: highest final concentrations of fatty acid achieved in well-emulsified reaction mixtures.

miniaturize the assay, which leads to a reduction of the handmade error and the costs.

Activity experiments on *C. rugosa* and *Bacillus*-related lipases validated the new method. Crl activity and linear range appeared similar when evaluated by both the colorimetric assay and by HPLC (Fig. 1). The activity found was nearly 10 times higher than that reported by Grippa et al. [28] using a different reaction mixture in the HPLC method (Fig. 1), and it was more similar to that reported by the manufacturer (704 μeq h^{-1} mg^{-1}, on olive oil). Therefore, using higher amounts of a better-emulsified substrate (nearly two times more) allows an increased detection of activity by both assays. Activity vs. substrate-concentration plots were also performed. As previously described [29], a very similar Michaelis–Menten plot was observed for Crl using both methods (Fig. 1C and D). Furthermore, the activities determined for the *Bacillus* lipases were 6–13 times higher than those obtained using the previous colorimetric method [15,22,25]. Only *Paenibacillus* sp. BP-23 EstA, a 5–10 times more active enzyme, showed almost no differences [11]. As shown above for the HPLC method, using higher amounts of a better emulsified substrate (2 times more) made the new assay more sensitive, and explains the increase in activity detected for the lipases with low activity. Activity vs. *p*-NPL-concentration assays confirmed also the typical Michaelis–Menten kinetics obtained previously for the *Bacillus*-related lipases using fluorogenic substrates [11,15,22,25,30].

The activity experiments reported above contributed to the validation of the new colorimetric method. The low intra- and inter-assay variations obtained and the fact that the method confirmed the low inhibitory effect of saturated fatty acids on Crl (Table 1A), previously described using HPLC [28] (Table 1C), confirmed the reliability of the system with regard to the inhibition assays. Small differences of inhibition were found, but they were less significant when the colorimetric method was performed using a reaction mixture (Table 1B) more similar to that of Grippa et al. [28]. The changes in the substrate used: 1 mM *p*-NPL (Table 1A and B), 0.46 mM β-NL (Table 1C), may explain the differences found in Crl inhibition. Both substrates are structurally related and yield similar activities for Crl at the same concentration. Therefore, the lower inhibition (greater IC_{50}) of Crl achieved in the colorimetric assays at high fatty acid concentrations (Table 1A and B) is probably due to the higher amount of substrate used, as fatty acids are known competitive inhibitors [18,19]. An increased ionic strength (Table 1B and C) seems to be also an important factor because it affects protein folding, the interaction between charged substances, and the emulsion properties by reducing the critical micellar concentration (CMC) of detergents [31]. Furthermore, the use of Ca^{2+} can reinforce the inhibition of a lipase, as is described for *P. acnes* lipase [1]. Additionally, changes in the detergent used: Triton X-100 (Table 1A and B), sodium taurocholate (Table 1C), were important to achieve good emulsions at higher substrate concentrations. Small pH variations: 7 (Table 1A) to 7.4 (Table 1B and C), showed a low effect on Crl as they were in the optimal pH range of the enzyme (6.5–7.5 Ref. [32]), but should be considered when other lipases or inhibitors are analyzed. Therefore, the composition of the reaction mixture should be taken into account when the inhibitions determined by different methods are compared.

The strong differences found when the new colorimetric assay was used to evaluate the effect of saturated fatty acids on *Bacillus*-related lipases (Table 2, Fig. 2) indicate that the results obtained from inhibition–activation assays cannot be extrapolated directly from one lipase to another. On the one hand, EstA and EstA1 carboxylesterases displayed a moderate–high inhibition by fatty acids (Table 2, Fig. 2). The inhibition observed could represent a negative feedback regulatory system controlled by product as suggested by the higher inhibition found by shorter fatty acids, which are closer to the enzyme optimal substrate. Such a regulation suggests that *Bacillus* cell-bound esterases could also be involved in the turnover of cell membrane lipids and lipid-anchored proteins. In this case, the enzyme product would avoid a high continuous activity on the cell membrane of these cell-bound esterases, which do not require interfacial activation to display activity. However, further studies are necessary to know if the inhibition found is competitive or/and specific, and to solve the regulation and physiological role of these enzymes.

On the other hand, *B. subtilis* LipA showed a different response to saturated fatty acids from cell-bound esterases, but also from LipB-like esterases (*B. megaterium* LipA and *B.* sp. BP-6 LipA). LipA showed a moderate activation at low fatty acid concentrations, and a moderate inhibition at medium–high concentrations, while LipB-like esterases were moderately (capric acid) and highly (myr-

istic and lauric acids) activated, and only slightly inhibited at high concentrations of capric acid. The higher activation of LipB-like proteins by long-chain lipids would allow their higher activity in the presence of these less specific substrates. It is known that fatty acids can bind to lipoprotein lipase in three different ways: at the active site, at specific sites, and cooperatively (hydrophobic interaction) [31]. For this reason, we hypothesize that the different activation achieved at low concentrations occurs by specific or highly cooperative binding of fatty acids to the protein surface, which is different for both lipases [16]. The inhibition could be due to a competition with the substrate for the active site because it appears at high fatty acid concentrations. The activation–inhibition found may be the result of the competition between these two different bindings.

To our knowledge, direct activation by fatty acids has not been described before for bacterial lipases. These compounds act usually as inhibitors, and only reactivation of pancreatic lipase–colipase complex inhibited by phospholipid–bile salts mixed micelles [33] and interfacial activation without an interface of fungal lipases in the presence of long acyl-CoA below their CMC [34] have been reported before. The possibility of an artifact was discarded, as the assay conditions were the same as those used for Crl and cell-bound esterases inhibition detection.

The differences in structure and regulation of *B. subtilis* LipA and LipB indicate different physiological functions [17], but also suggest that the *lipA* gene could have duplicated and evolved to obtain a protein (LipB) with the same core but different surface residues than LipA, and adapted to act at high concentrations of long-chain lipids and other situations in which LipA is poorly active. The fact that LipB expression is enhanced by compounds related to fatty acids like tributyrin and *n*-hexadecane [16], together with the high identity found among LipB-like genes [15], reinforces also the theory of a more recent origin of LipB and of its higher adaptation to lipid-rich environments. In conclusion, the activation by saturated fatty acids found in this work seems to be part of a more general regulatory system evolved to ensure a high use of carbon sources in nutrient-rich environments with strong microbial competition, and it could be related to the successful adaptation of *Bacillus* strains to many different habitats.

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Isolation of lipid- and polysaccharide-degrading micro-organisms from subtropical forest soil, and analysis of lipolytic strain *Bacillus* sp. CR-179

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ABSTRACT

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Aims: To isolate the micro-organisms from three soil samples obtained from a subtropical forest of Puerto Iguazú (Argentina), to analyse them for detection of the biotechnologically interesting enzymatic activities lipase, esterase, cellulase, xylanase and pectinase, and to identify the most active strain.

Methods and Results: A total of 724 strains were isolated using different culture media and temperatures, and 449 of them showed at least one of the hydrolytic activities pursued. Lipolytic activity of the lipid-degrading strains was further determined using MUF-butyrate and MUF-oleate as substrates. The alkalophilic strain CR-179, one of the most active for all the enzymatic activities assayed, was characterized and preliminarily identified by morphological, physiological and 16S rDNA tests, as a *Bacillus* sp. closely related to *Bacillus subtilis*.

Conclusions: Highly hydrolytic strains were isolated from all soil samples, suggesting the existence of a microbial community well-adapted to nutrient recycling. Strain CR-179, one of the most active, has been preliminarily identified as a *Bacillus* sp.

Significance and Impact of the Study: A collection of hydrolytic strains with high biotechnological potential was obtained. Presence of sequences codifying for a lipolytic system related to the *B. subtilis* group lipases was revealed by PCR for the best lipolytic strain.

Keywords: *Bacillus*, cellulase, esterase, lipase, pectinase, subtropical forest soil, xylanase.

INTRODUCTION

Micro-organisms or their enzymes are used in a wide range of biotechnological activities such as polymer hydrolysis, synthesis of value-added compounds, decontamination of soils, etc. Among these activities, the improvement of industrial processes using microbial enzymes is one of the most important fields of research because enzyme-catalysed reactions are highly efficient and selective, are less polluting, and usually require mild conditions and less energy, which leads to the lowering of costs (Cherry and Fidantsef 2003). Thus, there is an increasing interest for isolating new enzymes and new enzyme-producing strains for their use in industrial conversions (Cherry and Fidantsef 2003). Among these enzymes, lipases, esterases, cellulases, xylanases and pectinases play an important role in many biotechnological processes.

Lipases are glycerol ester hydrolases acting on long (true lipases) or short (esterases) acylglycerols to liberate fatty acids and glycerol (Jaeger *et al.* 1999). These enzymes catalyse also the reverse reaction, namely ester synthesis and transesterification (in nonaqueous systems), and they can catalyse stereoselective and regioselective reactions. Moreover, lipases and esterases show activity on a great variety of substrates, with no requirement for added cofactors. Thus, they are very interesting biocatalysts for industrial purposes such as detergency, flavour production, paper recycling,

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chemical synthesis, resolution of racemic mixtures, etc. (Jaeger *et al.* 1999).

Cellulases (endoglucanases, exoglucanases and β-glucosidases) are a group of enzymes that catalyse the hydrolysis of glucosidic bonds in cellulose, the most abundant polysaccharide in vegetable biomass. They are currently used in paper recycling, textile fibre treatment, alcohol and biofuel production, etc. (Bhat 2000).

(Endo)xylanases produce the hydrolysis of the main polyxylose backbone from xylans, which are the second most abundant vegetable polysaccharides. Some of the main industrial processes improved by xylanases are: removal of xylan and lignin from paper pulp, biofuel and dissolvent production, wine clearing and juice extraction (Beg *et al.* 2001).

Pectinases are a complex group of enzymes responsible for the degradation of pectin polysaccharides such as polygalacturonic acid (PGA). These enzymes are used in clarification, extraction and concentration of juice, tea and coffee fermentation, vegetable biomass liquefaction or textile fibre production (Kashyap *et al.* 2001).

The enzymatic properties and biotechnological applications described justify the increasing interest for finding new enzymes or new enzyme-producing strains with novel biotechnological potential, including the five enzymatic activities mentioned. Soils are a good source of enzyme-producing strains because they are nutrient-rich environments where there is a high proliferation of micro-organisms. Many of these micro-organisms are highly active in nutrient recycling, mainly in the degradation of vegetable polysaccharides such as lignocellulose and its phenolic-derived compounds. The microbial diversity of each soil depends on nutrient availability and several physicochemical properties related to the climate and type of soil (Atlas and Bartha 1998).

Here we report the isolation of indigenous bacterial and fungal strains from three soil samples obtained from a subtropical forest of Puerto Iguazú (Argentina). The isolated strains have been analysed to detect the biotechnologically interesting enzymatic activities mentioned above, and lipase activity of the most lipolytic strains has been determined. Furthermore, we have analysed the biochemical and physiological properties of the lipolytic strain CR-179, one of the most active isolates.

MATERIALS AND METHODS

Isolation and culture media

Three soil samples were collected from a subtropical forest in Iguazú rainfalls (Puerto Iguazú, Argentina). Soil no. 1 was a mixture of decomposing wood and surface soil, soil no. 2 was clayey material buried 20 cm from the surface, and soil no. 3 was surface soil with a high content in vegetable material. The soils were stored at room temperature in axenic conditions and protected from direct sun light until the isolation was performed.

Micro-organisms were extracted by suspending 1 g of each soil sample in 20 ml saline solution Ringer 1/4 (2·25 g NaCl, 0·105 g KCl, 0·12 g $CaCl_2$ per litre; pH 7·2; ADSA Micro, Pharmafaster SA, Barcelona, Spain). After 10 min vigorous stirring and additional sedimentation, two samples of 8 ml of the aqueous phase were collected. One of the samples was treated for 10 min at 80°C to isolate only spore-forming bacteria, and the other sample remained untreated in order to isolate spore-forming and nonspore-forming micro-organisms.

These samples were used for isolation of aerobic and facultatively anaerobic micro-organisms by means of serial dilutions. 0·1 ml of each dilution were spread on agar plates containing five different culture media and the resulting plates were incubated, in duplicate, at three different temperatures (20, 30 and 42°C). According to the micro-organisms pursued, the five culture media used were: Luria–Bertani (LB) agar plates [10 g bactotryptone (ADSA), 5 g yeast extract (ADSA), 10 g NaCl (Panreac Química SA, Castellar del Vallès, Spain), 15 g agar (ADSA), per litre; pH 7·0]; nutrient broth agar (ADSA Micro: 5 g peptone, 2 g yeast extract, 5 g NaCl, 1 g meat extract, 15 g agar, per litre; pH 7·4); Sabouraud-chloramphenicol agar [ADSA Micro: 5 g tryptone, 5 g meat peptone, 40 g D(+)-glucose, 0·5 g chloramphenicol, 15 g agar, per litre; pH 5·6]; Horikoshi-I agar [Horikoshi 1999; 10 g D(+)-glucose (Panreac), 5 g yeast extract, 5 g bactotryptone, 1 g K_2HPO_4 (Panreac), 0·2 g $MgSO_4 \cdot 7H_2O$ (Panreac), 10 g Na_2CO_3 (Panreac), 20 g agar, per litre; pH 10]; emulsified CeNAN-olive oil agar (ADSA Micro: 5·5 g peptone, 3·5 g yeast extract, 15 g agar, per litre; pH 7·5; plus 1% v/v 1° olive oil from Carbonell (Aceites Carbonell SA, Córdoba, Spain), and 0·0002% v/v Rhodamine B from Sigma).

For each medium and temperature of isolation, those colonies displaying different morphological properties were selected and their Gram stain performed. A pure culture for each strain was obtained and maintained using the same culture conditions of isolation.

Hydrolytic activity detection

The hydrolytic activities on tributyrin, olive oil, xylan, carboxymethyl cellulose (CMC) and PGA of the 724 strains isolated were screened by means of a direct test based on streaking the isolated strains on agar plates supplemented with the desired substrate and incubated at the isolation temperature of each strain. True lipase activity was detected after growth in emulsified CeNAN-olive oil agar, by the presence of pink/orange-coloured colonies and by

fluorescence emission under ultraviolet light (Kouker and Jaeger 1987; Ruiz *et al.* 2002). Esterase activity was detected by hydrolysis haloes visible around the colonies grown on emulsified CeNAN agar plates supplemented with 1% v/v tributyrin from Scharlau and 0·0002% v/v Rhodamine B (Prim *et al.* 2000). Xylanase activity was observed by direct appearance of hydrolysis areas surrounding the colonies after growth on xylan-agar plates (LB supplemented with 0·4% w/v oat spelt xylan from Sigma). Cellulase and pectinase activities were detected after growth on either CMC-agar plates (LB supplemented with 0·5% w/v CMC from Sigma) or PGA-agar plates (nutrient broth agar supplemented with 1% w/v PGA from Sigma), followed by congo red (Blanco *et al.* 1998) or HCl treatment (Soriano *et al.* 2000) respectively.

Lipolytic activity determination

Fluorescence emission of the 48 most active strains in emulsified CeNAN-olive oil or tributyrin agar plates was assayed by measuring the release of 4-methylumbelliferone (MUF) from MUF-derivative substrates produced by cell suspensions in phosphate buffer 50 mM pH 7 as previously described (Diaz *et al.* 1999).

Crude cell extracts and culture supernatants of the 29 strains showing the highest fluorescence emission in the previous assays were prepared as described before (Ruiz *et al.* 2002). Briefly, liquid cultures of the corresponding isolates were centrifuged for 10 min at 4000 *g*. The supernatants were recovered, whereas the pellets were suspended in 50 mM phosphate buffer (pH 7), sonicated and cleared by centrifugation for 10 min at 10 000 *g*. The resulting cleared cell lysates were recovered and considered as the cytoplasm fractions (cell extracts). Supernatants and cytoplasm fractions were used to determine the lipolytic activity of these strains on MUF-butyrate and MUF-oleate, as previously described (Prim *et al.* 2000; Ruiz *et al.* 2003). One unit of activity was defined as the amount of enzyme that released 1 μmol of MUF per minute under the assay conditions used. Protein concentration of the samples was determined using the Bio-Rad Protein Assay (Bio-Rad Laboratories SA, Cerdanyola del Vallès, Spain).

Bacterial identification of strain CR-179

Identification of the hydrolytic strain CR-179 was performed through morphological, physiological and molecular assays. Motility test, Gram, spore and other specific stains were performed as previously described (Sánchez 2004), and cells were observed at ×1000 under an Olympus B202 double beam optical microscope (Olympus Optical España SA, Barcelona, Spain). Transmission (TEM) and scanning (SEM) electron microscopy were performed at the Serveis Científico Tècnics of the University of Barcelona using cells from strain CR-179 grown in LB medium for 48 h at 42°C. Microscopes TEM 100 kV-Hitachi H6000 AB and SEM-Hitachi S-2300 were used (Hitachi High Technologies America Inc., Cerdañola del Vallès, Spain).

Determination of the physiological properties of strain CR-179 was performed according to the microbiological tests recommended for Gram-positive bacteria (Gordon *et al.* 1973), including, among other assays, aerobic or anaerobic growth, pH range of growth, methyl red and Vogues–Proskauer tests, oxidase, catalase, hydrolysis of casein, gas and acid production from lactose, use of citrate, nitrate reduction, indole production, hydrolysis of polysaccharides, etc. API 20-E and API 50CH galleries (Bio-Mèrieux, Marcy-l'Etoile, France) were also used.

Genomic DNA from strain CR-179 was isolated essentially as described in Sambrook *et al.* (1989) and used as a template for PCR amplification in a reaction mixture containing 1× PCR buffer (Biotools, Madrid, Spain), 250 μM of each deoxynucleoside triphosphate, 1·25 mM $MgCl_2$, 0·4 μl *Taq* Polymerase (Biotools), 12 pmol of primer Ty04F (5′-ATGGAGAGTTTGATCCTG-3′), 12 pmol of primer Ty06R (5′-TACCTTGTTACGACTT-3′), 50-100 ng DNA, and H_2O up to 50 μl. The mixture was subjected to an initial denaturing step consisting of 5′ at 95°C, followed by an amplification program consisting on 29 cycling periods of 30 s at 96°C, 30 s at 50°C and 2 min at 72°C. The purified PCR products were sequenced using a *BigDye*® Terminator V3·1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and the analytical system CEQ™ 8000 available at the Serveis Científico Tècnics of the University of Barcelona. Percentage similarity of the resulting 16S rDNA sequences was analysed through Blast (Altschul *et al.* 1997; http://www.ncbi.nlm.nih.gov/blast), and sequence alignments were performed using ClustalW Multalign on-line software (http://www.ebi.ac.uk/clustalW). Matrices of evolutionary distances were computed from the sequence alignment using the program MEGA2 (Sudhir *et al.* 2001). Distance matrices were performed according to the neighbour-joining algorithm and with the Jukes and Cantor (1966) model. To validate the reproducibility of the branching pattern, a bootstrap analysis was performed.

Detection of lipase-coding genes of strain CR-179

Detection of CR-179 lipolytic enzyme-coding genes was performed by PCR using consensus primers for *Bacillus* lipases central regions as previously described (Ruiz *et al.* 2002, 2003). Amplifications using *Bacillus subtilis* LipA-specific primers (Sánchez *et al.* 2002) and *B. subtilis lipB* (*yfiP*) primers (Ruiz *et al.* 2002) were also performed.

Nucleotide sequence accession number

The 16S rDNA sequence of strain CR-179 was submitted to the EMBL under accession number AJ821280.

RESULTS

Isolation of micro-organisms from three soil samples

Three samples of soil from a subtropical forest of Iguazú rainfalls (Puerto Iguazú, Argentina) were used for the isolation of native micro-organisms growing in an organic matter-rich environment, using the different culture conditions as described in *Materials and methods*. A total of 724 pure cultures of bacteria and fungi were obtained (Table 1). All soil samples showed similar values of colony-forming units per gram of soil (CFU $g^{-1} = 2–6 \times 10^5$; Table 1), independently of the culture medium and temperature used or the 80°C treatment, although important differences were observed in the morphology of the isolated colonies. Sabouraud-chloramphenicol medium was the exception because no growth was observed for the 80°C-treated samples, whereas the values obtained for the unheated samples were 10-fold lower than those obtained in the other culture media.

Screening of the enzymatic activities of the isolated strains

The hydrolytic activities of the 724 strains isolated were detected using a plate test for olive oil, tributyrin, xylan, cellulose and pectin degradation (see Materials and methods). Among the isolates analysed, 449 were active on one or more of the substrates evaluated, and 43 of them degraded all substrates. Table 2 shows the results with respect to each soil and substrate analysed. Nearly half of the strains displayed lipolytic activity (331 degraded olive oil, and 360 were active on tributyrin), whereas the number of strains active on polysaccharides was lower (114, 116 and 82 degraded xylan, cellulose and pectin respectively). Almost no differences were found for the isolation conditions with respect to the type of hydrolytic activities or the relative number of active strains obtained, with the exception of a slight predominance among the active strains of those isolated at 20 and 30°C.

The 76 strains bearing the highest hydrolytic activities were selected and stored for further characterization. Among them, strain CR-179, one of the most active isolates on all the substrates assayed, is shown in Fig. 1 as an example of the enzymatic activities evaluated.

Table 1 Number of viable g^{-1} and isolates from each soil

Soil	CFU g^{-1}* (total)	CFU g^{-1}* (fungi)	Isolates† (total)	Isolates† (fungi)
1	2×10^5	2×10^4	110	20
2	6×10^5	6×10^4	260	19
3	6×10^5	6×10^4	354	32
Total			724	71

*Colony-forming units (CFU) per gram of soil. Maximum obtained among all culture media and temperatures analysed. Total refers to bacteria plus fungi.
†Number of different strains isolated in pure culture.

Table 2 Screening of the degradative activities of soil isolates

Soil	Isolates	Olive oil	Tributyrin	Xylan	CMC*	PGA†
1	110	48	48	13	19	4
2	260	110	129	49	56	36
3	354	173	183	52	41	42
Total	724	331	360	114	116	82

*Carboxymethyl cellulose.
†Polygalacturonic acid.

Lipase activity determination

Lipolytic activity of the strains showing a good performance in hydrolysis of olive oil and/or tributyrin was subsequently analysed by measuring the fluorescence emission produced by cell suspensions acting on MUF-butyrate and MUF-oleate. Those 48 strains producing the highest fluorescence emission were chosen for further quantification of their lipolytic activity. Table 3 shows the activity results on MUF-butyrate and MUF-oleate produced by crude cell extracts and culture supernatants of the 29 selected strains. Activity on MUF-butyrate was higher than that on MUF-oleate for all strains, and CR-179 was the most active in general terms. This strain was by far the most active on MUF-butyrate (cell extracts: 1418·8 mU mg^{-1} protein, and 6·9 mU ml^{-1}; supernatants: 7·6 mU ml^{-1} of culture), and it was also very active on MUF-oleate (cell extracts). Other strains such as CR-290, CR-203, CR-445 (cell extracts) displayed also high activity on MUF-butyrate, whereas some strains such as CR-611 (cell extracts) and the fungal strains CR-31 (supernatants) and CR-563 (cell extracts) were very active on both MUF-butyrate and MUF-oleate (Table 3).

Selection and identification of strain CR-179

Strain CR-179 was isolated from soil no. 2 sample treated for 10 min at 80°C, and incubated under aerobic conditions on Horikoshi-I plates (pH 10) for 24 h at 42°C. This strain, producing high degradation of tributyrin, olive oil, xylan, cellulose and PGA (Fig. 1), and being the most active strain

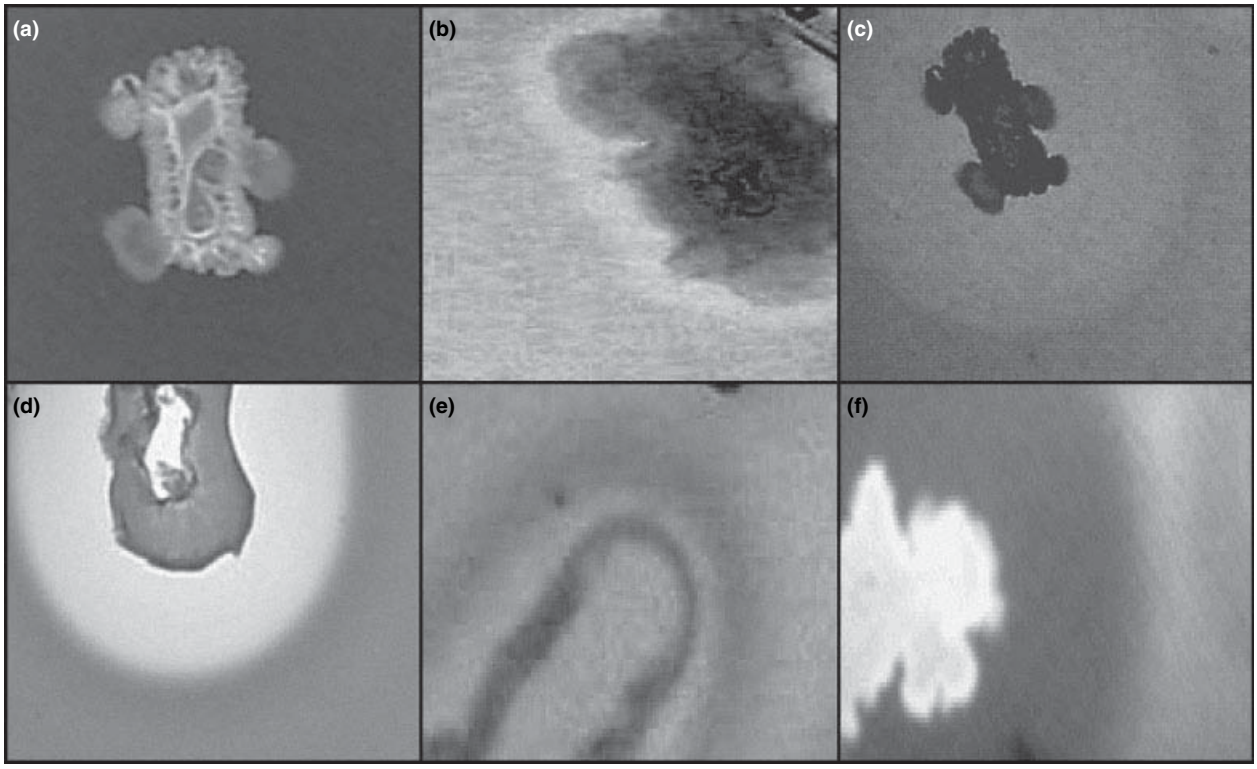

Fig. 1 Hydrolytic activities of strain CR-179 growing on agar plates supplemented with olive oil (a), tributyrin (b), xylan (c), carboxymethyl cellulose (d), polygalacturonic acid (e) and casein (f). Fluorescence emission (a) and haloes around the streak (b–f) are indicators of the biotechnologically interesting activities: lipase, esterase, xylanase, cellulase, pectinase and protease respectively

on MUF-butyrate and also highly active on MUF-oleate (Table 3), was chosen for further characterization.

Strain CR-179 produced white, round (diameter: 0·5 cm) but nonregular, crooked colonies with mucous material inside covered by an external coat. Gram and spore stains showed a Gram-positive rod with some pleomorphism, and many linear or branched chains frequently bound to a mucous matrix (Fig. 2a). CR-179 showed also an oval subterminal endospore slightly distending the mother cell. These morphological features were confirmed by SEM and TEM (Fig. 2b–d); motility test was positive.

CR-179 was able to grow at 30 and 42°C. It grew at 42°C on LB and nutrient broth agar plates in the pH range from 4·8 to 10·3, but it was unable to grow on minimal media supplemented with tributyrin, triolein or olive oil. This strain was positive/positive in the oxidation/fermentation of glucose assay (facultative anaerobic), and it was positive for oxidase, catalase, β-galactosidase, gelatinase, Voges–Proskauer and nitrate reduction tests. Furthermore, it was very active in the degradation of casein (Fig. 1f), indicating the presence of proteases among its hydrolytic enzymes. The strain was negative for the tests of methyl red, arginine dehydrolase, lysine and ornitine decarboxylase, urease, tryptophan deaminase, indole production, citrate utilization, hydrogen production from H_2S, and growth on MacConkey medium.

Carbohydrate degradation at 30°C for 48 h was positive for D-glucose, D-arabinose, D-fructose, D-mannose, mannitol, sorbitol, amygdalin, esculin, cellobiose and saccharose. It was negative for glycerol, erythritol, L-arabinose, ribose, D-xylose, L-xylose, adonitol, β-methyl-xyloside, galactose, L-sorbose, rhamnose, dulcitol, inositol, α methyl-D-mannoside, α methyl-D-glucoside, N-acetyl glucosamine, arbutin, salicilin, maltose, lactose, melobiose, trehalose, inulin, melizitose, D-rafinose, starch, glycogen, xylitol, β gentibiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate, 2-keto gluconate and 5-keto gluconate.

The results obtained through the morphological and biochemical tests assigned the strain CR-179 to the phenotypic group II of the genus *Bacillus* (*B. subtilis* group; Priest 1993), although no complete match in carbohydrate degradation was obtained with any previously defined *Bacillus* type strain species. The closest species were *Bacillus megaterium*, *Bacillus lentus*, *Bacillus stearothermophilus*, *B. subtilis* and *Bacillus pumilus*, which differed from CR-179 in the degradation of one (D-arabinose), two (D-arabinose and sorbitol), two (D-arabinose and maltose), three (D-arabinose,

Table 3 Spectrofluorimetric quantification of the lipolytic activity of the most active isolates

	MUF-butyrate			MUF-oleate		
	Cell extracts		SN*	Cell extracts		SN*
Isolates	mU mg^{-1} protein	mU ml^{-1}	mU ml^{-1}	mU mg^{-1} protein	mU ml^{-1}	mU ml^{-1}
Soil 1						
CR-3	12·9	0·8	1·2	0·910	0·050	0·004
CR-6	15·6	1·2	1·7	0·370	0·030	0·002
CR-31†	–	0·1	4·0	–	0·030	0·201
CR-39†	–	0·1	2·0	–	0·020	0·000
CR-40†	23·9	1·8	0·0	0·090	0·070	0·000
CR-53	9·7	0·3	1·1	1·230	0·040	0·038
CR-56†	23·6	2·0	0·8	0·960	0·080	0·000
CR-64	6·9	0·3	0·2	0·000	0·000	0·000
CR-70†	15·0	1·1	0·0	1·310	0·090	0·000
CR-89†	12·1	0·3	1·6	0·080	0·000	0·003
CR-95†	5·6	0·2	1·0	0·480	0·010	0·016
CR-105	36·2	2·7	2·8	0·000	0·000	0·021
CR-109	30·5	0·6	0·9	0·450	0·010	0·009
Soil 2						
CR-179	1418·8	6·9	7·6	4·650	0·020	0·003
CR-203	84·0	2·4	0·4	0·270	0·010	0·000
CR-208	65·0	1·1	0·3	0·420	0·010	0·000
CR-237	5·7	0·2	0·3	0·250	0·010	0·002
CR-290	364·6	3·9	7·0	1·100	0·010	0·002
CR-313†	18·7	0·0	0·1	0·000	0·010	0·000
CR-377	–	0·3	0·4	5·020	0·150	0·002
Soil 3						
CR-445	54·5	2·0	0·9	0·260	0·010	0·000
CR-479	25·9	0·8	0·3	0·000	0·000	0·000
CR-518	19·2	0·6	1·1	0·000	0·000	0·000
CR-522	24·6	0·6	1·1	0·000	0·000	0·015
CR-563†	22·5	2·1	0·8	9·770	0·910	0·006
CR-611	32·5	2·2	0·3	6·450	0·450	0·012
CR-649	27·2	0·4	0·4	0·720	0·010	0·002
CR-653	11·6	0·2	0·2	0·000	0·000	0·002
CR-699	15·8	1·2	0·3	2·980	0·230	0·008

The activity of all supernatants, and some cell extracts (–) are not expressed as mU mg^{-1} protein, as protein content was undetectable by the Bradford assay.

Results are the mean of two independent assays, each one performed in duplicate. The standard deviations obtained ranged from 5 to 10% of the corresponding mean values.

*Cell culture supernatants.

†Fungal isolates.

ribose, and maltose) and three (D-arabinose, arbutin, and salicilin) carbohydrates respectively.

Thus, traditional methods were followed by 16S rDNA sequence analysis. The sequence of 1453 bp obtained displayed 100% identity (ClustalW) with *Bacillus* sp. WL-3, and 98–99% identity with other strains of *Bacillus* RNA group I (Ash *et al.* 1991). The closest type strain species (99% identity) were *B. subtilis* 168, and *Bacillus vallismortis* DSM11031 (Fig. 3a).

As stated by Goto *et al.* (2000), species of *Bacillus* genus can be better classified using only the hypervariable (HV) region of the 16S rDNA (275 bp) because this region emphasizes better the phylogenetic distances. HV region of CR-179 16S rDNA showed 100% identity with *Bacillus* sp. WL-3 and *B. subtilis* BS-S3, whereas *B. subtilis* strains 168 and NCDO1769 were the closest type strain species (99% identity) in the phylogenetic tree obtained (Fig. 3b).

Therefore, the morphological, biochemical and 16S rDNA analysis performed with CR-179 allowed us to identify the isolated strain as *Bacillus* sp. CR-179, although it could not be assigned to any known *Bacillus* species.

Detection of lipase-coding genes of strain CR-179

The lipolytic activity of strain CR-179, and its assignation to the genus *Bacillus*, led us to perform PCR experiments using consensus primers for the *Bacillus* lipases conserved central region in order to identify CR-179 lipolytic enzymes. A partial sequence of 271 bp showing 73% identity (ClustalW) to *B. subtilis* LipA (Dartois *et al.* 1992) was obtained. However, the complete sequence could not be obtained using LipA specific primers. As previously reported (Ruiz *et al.* 2003), amplification using *B. subtilis* LipB primers confirmed the presence in this strain of an esterase showing 98–100% identity to *B. subtilis* LipB (Eggert *et al.* 2000), *B. megaterium* LipA (Ruiz *et al.* 2002) and *Bacillus* sp. BP-6 LipA (Ruiz *et al.* 2003).

DISCUSSION

Native micro-organisms growing in an organic matter-rich environment were isolated, using different culture media and temperature conditions, from three soil samples collected from a subtropical forest of Puerto Iguazú (Argentina). The values of total CFU g^{-1} obtained were 2–6 $\times$ 10^5, 10-fold higher than those corresponding to fungal CFU g^{-1} of soil (Table 1). From the isolated colonies considered as different on the basis of their morphology, 724 sporulated and nonsporulated, aerobic and facultative anaerobic, quimioheterotrophic, bacterial and fungal strains were obtained in pure culture.

The quantitative results obtained are in agreement with those previously reported (10^5–10^9 CFU g^{-1} dry weight; Atlas and Bartha 1998) although some considerations concerning the type and number of micro-organisms isolated should be taken into account. The preparation of samples (extraction and fragmentation of the microcolonies stuck to soil particles, breaking of hyphae or sporangia, etc.) can affect the results obtained (Alexander 1977). Moreover, there is not a culture medium or a temperature suitable for

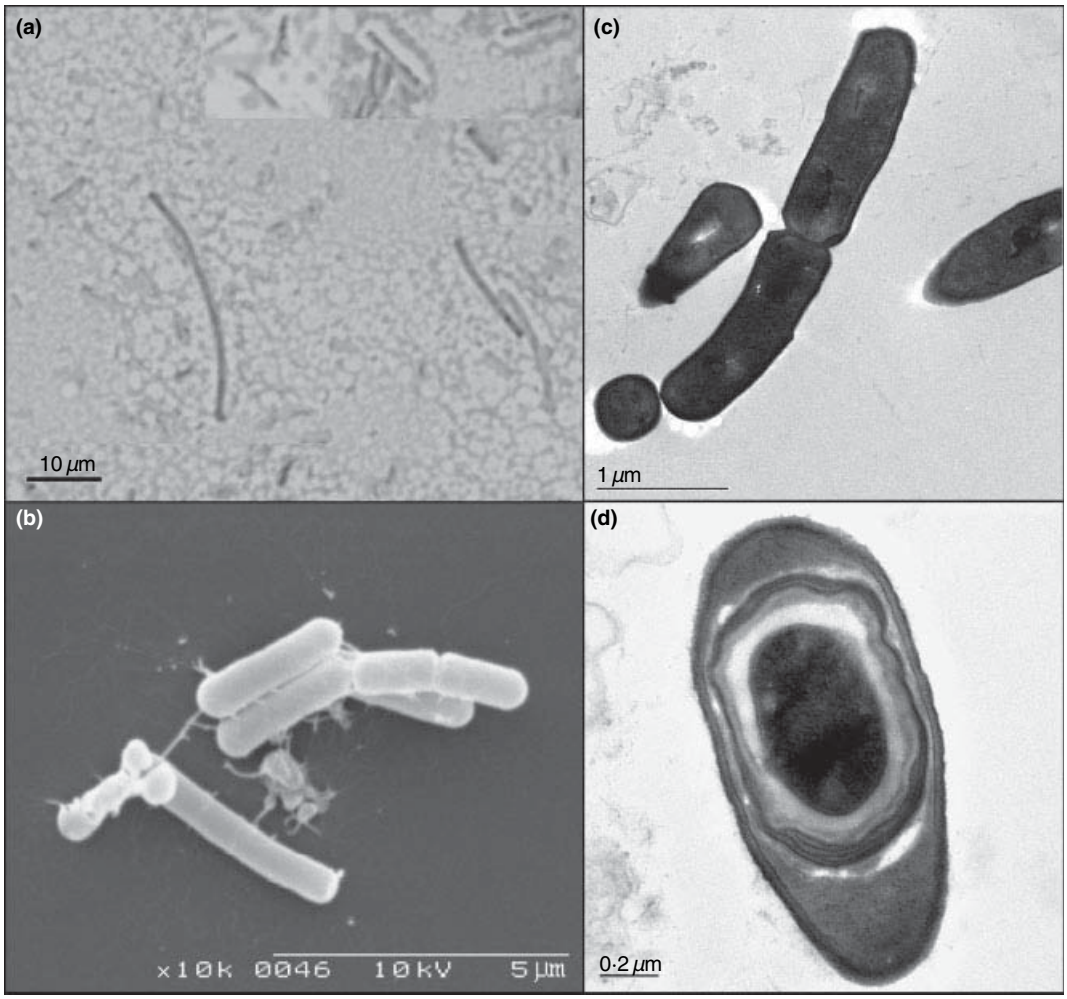

Fig. 2 Microphotographs of strain CR-179 obtained by optical (a), scanning (b) and transmission (c, d) microscopy. A certain degree of pleomorphism (a–c) and a mucous matrix around the cells (a) or binding several cells (b) can be observed. Details of cell division (c) and a spore inside a cell (d) are also shown

all micro-organisms, although different media and temperatures were used to reduce this limitation.

All 724 isolated strains obtained in pure culture were analysed to detect five biotechnologically interesting enzymatic activities: lipase, esterase, xylanase, cellulase and pectinase. Among the isolates, 43 showed all the activities and have, therefore, a high biotechnological potential, whereas 449 isolates showed one or more of these activities (Table 2). Many strains were active on polysaccharides: 114 degraded xylan, 116 hydrolysed cellulose and 82 degraded pectin (Table 2), as is common for soils rich in vegetable biomass (López *et al.* 1998; Soares *et al.* 1999; Semedo *et al.* 2000). Soil strains with lipolytic activity are also frequent (Prim *et al.* 2001; Ruiz *et al.* 2003). However, their high number with respect to that of polysaccharide-degrading strains (331 degraded olive oil and 360 were active on tributyrin plates; Table 2) could seem surprising for soils rich in wood, dead leaves, etc., unless considering that vegetable polysaccharides are bound and covered by a high amount of very complex lipid compounds (named 'pitch' in paper industry) such as waxes, phenolic compounds, and others, which have to be degraded to obtain cellulose and the other structural polysaccharides (Williamson *et al.* 1998; Jaeger *et al.* 1999). Moreover, presence of lipolytic activity in soil micro-organisms has been correlated to degradation of toxic compounds (Margesin *et al.* 1999). The results obtained indicate that the microbial communities of the soils analysed display a high complexity. Isolates showing all the enzymatic activities tested would be among the main responsible for the degradation of vegetable material, probably acting in cooperation with more specific strains to perform a synergistic degradation of vegetable biomass.

Seventy-six strains, bearing the highest hydrolytic activities, were selected and stored. Those active on

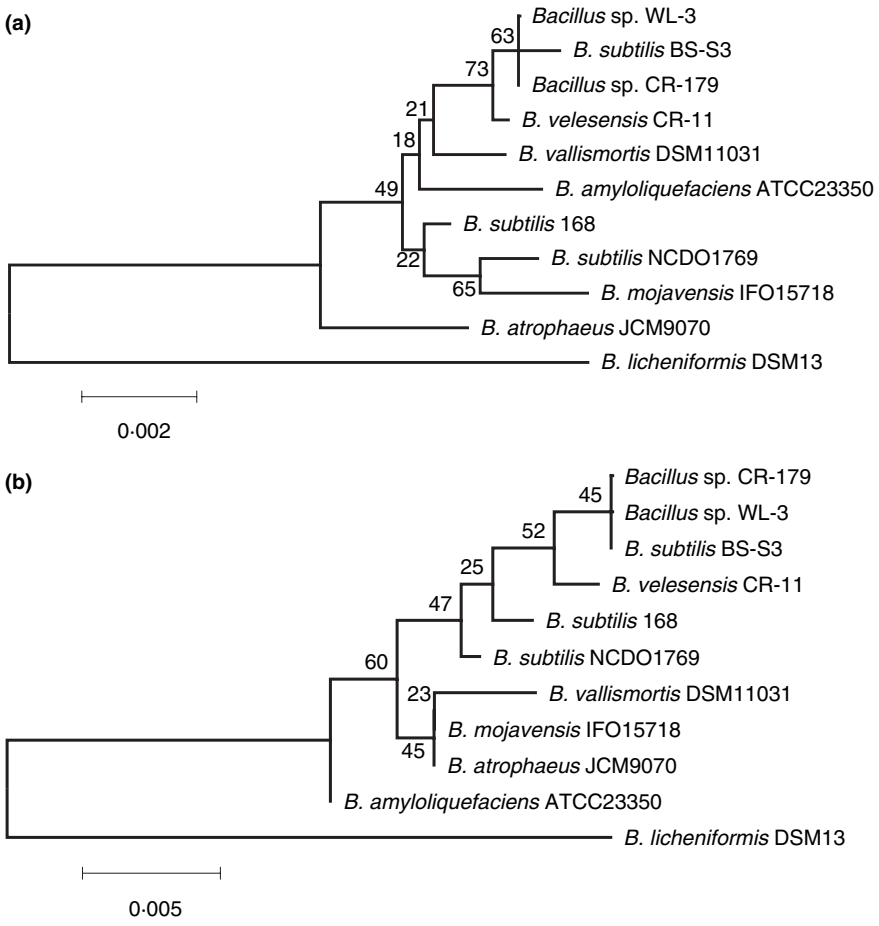

Fig. 3 Phylogenetic trees based on whole 16S rDNA sequences (a) and the hypervariable regions of 16S rDNA sequences (b) of strain CR-179, and its closest strains and *Bacillus* type strain species. The bar indicates the 0·2% (a) and 0·5% (b) estimated difference in nucleotide sequences. Number on the branches are bootstrap values. The accession numbers of the 16S rDNA sequences used to perform the phylogenetic trees are the following: *Bacillus* sp. WL-3 (AY601723), *B. subtilis* BS-S3 (AY583216), *B. subtilis* 168 (Z99104), *B. subtilis* NCDO1769 (X60646), *B. amyloliquefaciens* ATCC23350 (X60605), *B. vallismortis* DSM11031 (AB021198), *B. licheniformis* DSM13 (X68416), *B. mojavensis* IFO15718 (AB021191), *B. atrophaeus* JCM9070 (AB021181), *B. velesensis* CR-11 (AY605932)

polysaccharides are being identified, and their enzymatic activities characterized. As there is an enormous interest for lipases and esterases acting on vegetable compounds in biotechnology, for example, for the treatment of vegetable biomass to release the phenolic compounds used in flavour production (Williamson *et al.* 1998), the lipolytic activity of the lipid-degrading strains was subsequently quantitated to select those more active.

A preliminary determination of lipolytic activity of these strains, performed using cell suspensions, allowed us to select the most active 29 strains for a further quantification of their lipolytic activity using MUF-derivative substrates. The activities obtained (Table 3) were higher on MUF-butyrate than on MUF-oleate for all strains, and they revealed some differences with the results obtained by plate assays, probably because of the fact that cell extracts are very rich in esterases, even in secretory micro-organisms. Among the strains analysed, CR-179 was the most active in general terms. Other strains produced also a high activity on MUF-butyrate (CR-290, CR-203 and CR-445), whereas some strains such as CR-611, and the fungal strains CR-31 and CR-563 were very active on both MUF-butyrate and MUF-oleate (Table 3).

These highly lipid-degrading bacterial and fungal strains and their lipases and esterases are being identified and characterized in our laboratory. Among them, the alkalophilic strain CR-179, the most active on MUF-derivative substrates, and one of the most active in the degradation of tributyrin, olive oil, xylan, cellulose and PGA (Fig. 1) has been preliminarily identified in this work.

Strain CR-179 was isolated from soil sample no. 2, treated for 10 min at 80°C, and incubated on Horikoshi-I plates at 42°C. The morphological, biochemical and 16S rDNA analysis performed allowed us to identify it as a member of phenotypic group II (*B. subtilis* group; Priest 1993) and RNA group I (Ash *et al.* 1991) of the genus *Bacillus*. The strain is closely related to *B. subtilis*, although it could not be assigned to any known *Bacillus* species. Therefore the strain remains named as *Bacillus* sp. CR-179, up to present.

Bacillus species are aerobic, Gram-positive, endospore-forming, rod-shaped bacteria commonly found in soil, water sources or associated with plants (Priest 1993). They are an important source of industrial enzymes such as cellulases (Ferrari *et al.* 1993). In addition, several *Bacillus* lipases

showing a high biotechnological potential have been recently described. In *B. subtilis* and related species the presence of a conserved lipolytic system that ensures a high utilization of carbon sources in nutrient-rich environments with strong microbial competition has been revealed. These lipolytic systems are commonly constituted by a cell-bound esterase, a secreted lipase and a secreted esterase (Ruiz *et al.* 2004). The existence in *Bacillus* sp. CR-179 of a lipase-coding gene showing 73% identity to *B. subtilis* LipA secreted lipase (Dartois *et al.* 1992), and of an esterase-coding gene showing 98–100% identity to *Bacillus* secreted esterases (Ruiz *et al.* 2003), indicates the presence of the mentioned lipolytic system, although additional lipases or esterases may exist in this strain in view of its high lipid-degrading activity. Therefore, further assays to perform a complete characterization of the lipolytic system of strain CR-179 are in progress. Also, its polysaccharide-degrading system is under investigation, as far as the high biotechnological potential of the strain is concerned.

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Identification of a lipase-producing *Rhodococcus* soil isolate

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Running Headline: *Rhodococcus* lipolytic strain

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ABSTRACT

Subtropical soil microbial isolates were screened for carbohydrate, tributyrin, or olive oil hydrolysis using agar plates supplemented with the corresponding substrates. A heterotrophic, aerobic, Gram-positive strain displaying activity on tributyrin was selected and further characterised. Analysis of the morphological and physiological traits of the strain placed it as a member of the genus *Rhodococcus*. Further 16S rDNA sequencing revealed a 99% identity to *Rhodococcus erythropolis*. The strain displayed lipolytic activity on short chain-length fatty acid-derivative substrates and showed the highest activity at the cell extract fractions. Zymogram analysis confirmed the presence of an intracellular lipolytic enzyme of ca. 60 kDa. Presence of an intracellular lipolytic enzyme in *Rhodococcus erythropolis* indicates that the strain could be of interest for biotechnological applications.

Keywords: *Rhodococcus*, lipase, esterase, soil, actinomycete

INTRODUCTION

Soil is an nutrient-rich environment where large amounts of microorganisms can proliferate. Most of these microorganisms contribute to the recycling of elements on earth by means of their capacity to convert biomass into the corresponding organic or inorganic derivatives. Bacteria, fungi, algae, protozoa and viruses are usually found in soil, where they are distributed according to their role, adaptative capacity and permanence under the specific soil conditions (Atlas and Bartha, 2001). They usually form a robust community capable of surviving and functioning under extremes of temperature, water availability, pH, energy resources, nutrient availability and salt concentration (Smith and Smith, 2001).

The number of bacterial cells in soil can be extremely high, reaching values of 10^8 viables $\cdot$ g^{-1} of dry weight. Certain bacterial groups bearing special structural or metabolic capacities are representative inhabitants of soil. This is the case of endosporulated bacteria or actinomycetes, the latter representing from 10 to 30% of the total soil bacteria (Alexander, 1977). In general, actinomycetes are able to degrade complex polymers, thus playing a relevant role in biomass conversion. These hydrolytic activities contribute to increase their adaptative capacity, and allow colonisation of habitats rich in complex nutrients where other microorganisms using simpler substrates could not grow (Kanaly and Harayama, 2001; Murakami et al., 2004). For this degradative purpose, actinomycetes display a wide range of enzymatic activities (Donadio et al., 2002), including the presence of lipases in members of the genera *Streptomyces*, *Mycobacterium, Propionibacterium* or *Arthrobacter* (Sommer et al., 1997; Miskin et al., 1997; Johri et al., 2001; Vujaklija et al., 2002). In general, presence of lipase activity in these microorganisms has been correlated to their ability for biocide or toxic compound degradation (Bell et al., 1998).

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). Ester hydrolysis is mediated by a nucleophilic attack in a charge-relay system, whereas in non-aqueous systems, lipases can catalyse the reverse reaction, namely ester synthesis and transesterification (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 et al., 1999).

We report here the isolation and characterisation of a bacterial strain obtained from a subtropical soil in Puerto Iguazú (Argentina). The strain, displaying lipase activity, was identified as *Rhodococcus erythropolis* CR-53 by means of microscopy, physiological and molecular assays. The wide range of chemicals degraded or transformed by rhodococci makes them actually or potentially useful in environmental or industrial biotechnology, as does their ability to perform multiple synthesis reactions (Bell et al., 1998). The applied and ecological features that distinguish rhodococci justify the increasingly recognised commercial potential of the members of this genus.

MATERIALS AND METHODS

Isolation and culture media

Three soil samples were collected from a subtropical area in Puerto Iguazú (Argentina) and stored at room temperature in axenic conditions, protected from direct sun light. Microorganisms were extracted by suspending 1 g of each soil sample in 20 ml Ringer¼ (ADSA Micro: 2.25 g NaCl, 0.105 g KCl, 0.12 g $CaCl_2$ per L). After 10 min vigorous stirring and additional sedimentation, 8 ml of the aqueous phase were collected and used for isolation of total aerobic, sporulated or facultatively anaerobic microorganisms by means of serial dilutions. 0.1 ml of each dilution were spread on agar plates containing different culture media according to the group of microorganisms pursued, and the resulting plates were incubated under different temperature and pH conditions.

The culture media used for isolation, screening and culture growth were: LB (10 g bactotryptone, 5 g yeast extract, 10 g NaCl, per L, pH 6.8), Nutrient broth agar (ADSA Micro: 5 g peptone, 2 g yeast extract, 5 g NaCl, 1 g meat extract, 15 g agar, per L, pH 7.2), Sabouraud-chloramphenicol agar (ADSA Micro: 5 g triptone, 5 g meat peptone, 40 g D(+)-glucose, 0.5 g chloramphenicol, 15 g agar, per L, pH 5.6), Horikoshi/I agar (Horikoshi, 1999; 10 g D(+)-glucose, 5 g yeast extract, 5 g bactotryptone, 1 g K_2HPO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 10 g Na_2CO_3, 20 g agar, per L, pH 10), Emulsified CeNAN-olive oil agar (ADSA Micro: 5.5 g peptone, 3.5 g yeast extract, 15 g agar, per L, plus 1% v/v 1º olive oil from Carbonell, and 0.0002% v/v Rhodamine B from Sigma), Emulsified CeNAN-tributyrin or triolein agar (ADSA Micro: 5.5 g peptone, 3.5 g yeast extract, 15 g agar, per L, plus 1% v/v tributyrin [or triolein] from Scharlau, and 0.0002% v/v Rhodamine B from Sigma), Xylan-agar (LB supplemented with 0.4% w/v oat spelt xylan from Sigma), CMC-agar (LB supplemented with 0.5% w/v carboxymethyl cellulose [CMC], from Sigma), and Pectin-agar (LB supplemented with 1% w/v polygalacturonic acid [PGA], from Sigma).

Hydrolytic activity detection

Pure cultures were obtained for 724 isolates, and their hydrolytic activities on tributyrin, olive oil, xylan, CMC and PGA were screened by means of a simple, direct test, based on streaking the isolated strains on agar plates supplemented with the desired substrate (Blanco et al., 1998; Blanco et al., 1999; Soriano et al., 2000; Prim et al., 2000). True lipase activity was detected by the presence of pink/orange-coloured colonies and fluorescence emission after growth in CeNAN-olive oil or CeNAN-triolein agar (Kouker and Jaeger, 1987; Ruiz et al., 2002). Esterase activity was detected on CeNAN-tributyrin agar plates, where hydrolysis haloes were visible around the colonies (Prim et al., 2000). Xylanase activity was observed by direct appearance of hydrolysis areas surrounding the colonies after growth on Xylan-agar plates (Blanco et al., 1999). Cellulase and pectinase activities were detected after growth on either CMC or PGA-agar plates followed by Congo Red (Blanco et al., 1998) or HCl treatment (Soriano et al., 2000), respectively.

Bacterial identification

Identification of selected strain CR-53 was performed through morphological, physiological and molecular assays. Gram, spore and acid fast-specific stains were performed as previously described (Sánchez, 2004), and cells were observed at 1000X under an Olympus B202 double beam optical microscope. Transmission (TEM) and scanning (SEM) electron microscopy was performed at the Serveis Científico Tècnics of the University of Barcelona, using cells from strain CR-53 grown in LB medium at 20ºC for 48 h. Microscopes TEM 100 kV-Hitachi H6000 AB and SEM-Hitachi S-2300 were used.

Determination of the physiological properties of strain CR-53 was performed according to the microbiological tests recommended for Gram-positive bacteria and actinomycetes (Gordon et al., 1973; Goodfellow et al., 1998), including growth temperature, aerobic or anaerobic growth, methyl-red and Vogues Proskauer tests, presence of oxidase and catalase, hydrolysis of urea or gelatin, gas and acid production from several carbohydrates including D-glucose, L-arabinose, D-xylose, D-manitol, or

D-fructose, use of citrate, nitrate reduction, indol production, or xylan, cellulose and polygalacturonic acid hydrolysis. API 20E and API 50CH galleries (BioMèrieux, Roche) were also used for confirmation.

Genomic DNA from strain CR-53 was isolated and used as a template for amplification of 16S rDNA, using universal primers, as described (Rainey et al., 1996). The purified PCR products were sequenced using a *BigDye*® Terminator V3.1 Cycle Sequencing Kit (Beckman-Coulter) and the analytical system CE™8000, available at the Serveis Científico Tècnics of the University of Barcelona. Homology of the resulting 16S rDNA sequence was analysed through BLAST (Altschul et al., 1997; http://www.ncbi.nlm.nih.gov/blast), and sequence alignments were performed using ClustalW Multalign on-line software (http://www.ebi.ac.uk/clustalw). Evolutive distances and the phylogenetic trees were established according to Felsenstein (1993).

Lipase 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 4-methylumbelliferone (MUF) from MUF-derivative substrates was measured as previously described (Prim et al., 2000). One unit of activity was defined as the amount of enzyme that released 1 μmol of MUF per minute under the assay conditions used. Cell extract or supernatant proteins were separated on 10% SDS-PAGE gels. Following zymogram analysis (Diaz et al., 1999), the gels were subsequently stained with Coomassie Brilliant Blue R®-250, and protein bands visualised.

Nucleotide sequence accession number

The 16S rDNA sequence of strain CR-53 was submitted to the EMBL under accession number AJ786263.

RESULTS

Isolation of strain CR-53

Three samples of subtropical soil from Puerto Iguazú (Argentina) were used for the isolation of autoctonous microorganisms growing in an organic matter-rich environment. A total of 724 pure cultures of bacteria and fungi were obtained and their hydrolytic activities assayed (see Materials and Methods). Those strains bearing some of the tested hydrolytic activities were stored for further characterisation. Strain CR-53, described in this work, was isolated from soil sample #1, a soil rich in small pieces of wood and wood powder coming from the decomposition of a decaying tree. The strain grew in LB medium at 20ºC, pH 6.8 and displayed hydrolytic activity on tributyrin (not shown). Colonies from strain CR-53 displayed clear hydrolysis haloes plus a pink/orange color after growth on CeNAN-tributyrin agar plates supplemented with rhodamine, showing fluorescence emission as a result of tributyrin hydrolysis (Kouker and Jaeger, 1987). However, no activity was observed on olive oil or triolein-supplemented agar plates, neither on xylan, polygalacturonic acid or CMC (not shown). Attending to the capacity of strain CR-53 to hydrolyse short fatty acid-containing triacylglicerides, the presence of an esterase was suspected and the strain was selected for identification and characterisation of its lipolytic system.

Identification of strain CR-53

Growth of strain CR-53 was achieved on LB media after 3-day incubation at 20ºC, pH 6.8, under aerobic conditions. The strain produced rough, creamy, mucoid colonies that tended to spread on the plates. After specific Gram, acid-fast and spore stains, strain CR-53 was identified as a non-sporulated, acid-fast negative, elementary-branching Gram-positive rod with a certain degree of snapping division (Figure 1), a frequent feature found among actinomycetes (Goodfellow et al., 1998). This morphology was confirmed by both, scanning and transmission electron microscopy, where the snapping division of the strain was clearly demonstrated (Figure 1). The strain was nonmotile, catalase and urease positive, sensitive to lysozyme, and was

negative for alcohol dehydrogenase, lysine or ornitine decarboxylase, arylsulfatase, lactose, citrate, indol, Vogues-Proskauer, and gelatinase, showing no gas production from H_2S. It was also unable to degrade cellulose, polygalacturonic acid or xylan. The results obtained through the biochemical tests performed assigned the strain to the genus *Rhodococcus* (Goodfellow and Alderson, 1977), although no complete match in carbohydrate degradation was obtained with any previously defined *Rhodococcus* type strain species (Goodfellow et al., 1998). Table 1 shows some of the most relevant results obtained from sugar utilisation in comparison with those from close *Rhodococcus* species, the highest similarity being found to *R. erythropolis*^{T} ATCC 4277.

As previously stated by other authors, identification of rhodococci to species level is difficult by traditional methods (Goodfellow, 1989). For this reason, microscopic examination and physiological tests were followed by 16S rDNA sequence analysis. The 1367 bp nucleotide sequence of the two strands from strain CR-53 16S rRNA gene was obtained for bacterial identification. Strain CR-53 displayed a high level (99%) of 16S rDNA sequence identity with other strains described as *R. erythropolis* (Katsivela et al., 1999), with *R. erythropolis*^{T} ATCC 4277, and with a number of unidentified *Rhodococcus* species (BLAST, http://www.ncbi.nlm.nih.gov/blast). The phylogenetic trees obtained by comparison of the calculated DNA sequence to those of other type strain species of the genus *Rhodococcus* (Figure 2) confirmed the proximity of strain CR-53 with *R. erythropolis*, allowing us to identify the isolated strain as *Rhodococcus erythropolis* CR-53.

Analysis of Rhodococcus erythropolis *CR-53 lipolytic system*

Concentrated culture supernatants and cell extracts from *Rhodococcus erythropolis* CR-53 were tested for lipolytic activity on MUF-derivative substrates (Prim et al., 2000). Cell extracts from the strain efficiently hydrolysed MUF-butyrate (5.12×10^{-4} units/ml culture), whereas activity at the supernatant fraction was much lower (1.08×10^{-4} units/ml culture), suggesting a cytosolic location of the enzyme(s). The strain displayed maximum activity on short-chain fatty acid-derivative substrates (MUF-butyrate), while activity on esters of long chain-length fatty acids like MUF-

oleate was almost negligible (1.54 x 10^{-7} units/ml culture). The preference of the lipolytic enzyme(s) produced by the strain for short-chain length substrates would be in agreement with the behaviour described for most esterases (Jaeger et al., 1999).

Activity of 50-fold concentrated supernatants and cell extracts from *Rhodococcus erythropolis* CR-53 was also analysed by zymogram on SDS-PAGE gels, using MUF-butyrate as a substrate (Diaz et al., 1999). A ca. 60 kDa band bearing activity on MUF-butyrate was detected in *R. erythropolis* CR-53 cell extracts (Figure 3), while no activity bands were observed in concentrated culture supernatants of the strain.

The size, substrate specificity and location of the 60 kDa activity band indicate that strain *Rhodococcus erythropolis* CR-53 codes for at least one intracellular lipolytic enzyme, probably an esterase that could be of interest for biotechnological or environmental applications.

DISCUSSION

Strain CR-53, isolated from a subtropical soil in Puerto Iguazú (Argentina) and described in this work, was selected among other microbial isolates because of its lipolytic activity and morphological properties. According to the results obtained from morphogenetic, metabolic and phylogenetic assays, the strain was identified as *Rhodococcus*, although some differences in hydrocarbon utilization were found with respect to the closest type strain *R. erythropolis*^{T} ATCC 4277 (Goodfellow et al., 2002). However, phylogenetic analysis of 16S rDNA contributed to asign the strain to this species, being named *Rhodococcus erythropolis* CR-53.

Rhodococci are aerobic, Gram positive actinomycetes of high G+C content, capable of morphological differentiation in response to their environment (Goodfellow et al., 1998). These widely occurring organisms are of considerable environmental and biotechnological importance due to their broad metabolic diversity and array of unique enzymatic capabilities (Bell et al., 1998). They exhibit the ability to convert a wide range of organic compounds, being able to degrade various chemical pollutants such as simple hydrocarbons, aromatic hydrocarbons, nitroaromatics, chlorinated polycyclic aromatics such as polychlorinated biphenyls (PCBs), and other recalcitrant toxic pollutants (Bell et al. 1998; Yoon et al., 2000). The enzymatic activities displayed by most Rhodococci are of interest to the pharmaceutical, environmental, chemical and energy sectors (Jones et al., 2004).

Strain *R. erythropolis* CR-53 was selected and further characterised because of its ability to degrade lipidic compounds. The strain displayed preference for short-chain fatty acid-derivative substrates, and a 60 kDa band with activity on MUF-butyrate could be detected on zymograms after SDS-PAGE separation of concentrated cell extracts. The presence of a single activity band with the size of most Gram-positive bacterial esterases suggests that *R. erythropolis* CR-53 produces an intracellular lipolytic enzyme with activity on MUF-butyrate, similar to family VII bacterial esterases (Arpigny and Jaeger, 1999), and present in many Gram-positive bacteria (Prim et al., 2000; Prim et al., 2001). On the basis of cellular location and substrate specificity, we named the putative esterase from *Rhodococcus erythropolis* CR-53 EstAVII. To our knowledge,

presence of a hypothetical esterase in a *Rhodococcus* species has been demonstrated here for the first time. The enzyme described could play an important adaptative role, contributing to favour growth under certain conditions, thus allowing colonisation of lipid-rich habitats like those containing decomposition organic matter or oil-contaminated environments (Bell et al., 1998). The lipid-hydrolising activity shown by *R. erythropolis* CR-53 indicates that the strain could be used for field or biotechnological applications requiring the presence of an esterase, as well as in steroid modification or enantioselective synthesis (Larkin *et a*l., 1998). Further studies are being conducted to perform the cloning and characterisation of the enzyme in order to evaluate its biotechnological possibilities.

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Table 1: Characteristics that distinguish strain CR-53 from close *Rhodococcus* type strain species

Substrate	**CR-53 - EB/R/C**	*R. erythropolis* ATCC 4277 EB/R/C	*R. marinonascens* DSM 43752 H/R/C	*R .rhodnii* DSM 43336 EB/R/C	*R. ruber* DSM 43338 H/R/C	*R. rhodochrous* DSM 43241 EB/R/C
Aesculin	+	+	+	-	-	+
D-glucose	+	+	+	+	+	-
D-fructose	+	+	+	-	+	+
Galactose	-	-	-	/	/	-
Glycerol	+	+	+	-	+	+
Inositol	+	+	+	-	-	-
Maltose	-	+	-	-	+	+
Mannitol	+	+	-	+	+	+
Rhamnose	-	-	-	-	-	-
Ribose	±	+	-	-	-	-
Sorbitol	+	+	+	+	+	+
Sucrose	-	+	-	+	+	+
Trehalose	+	+	-	-	+	+
β-methyl-xyloside	-	-	w	w	-	-

Symbols: +: positive; -: negative; /: not determined; ±: variable; w: weakly positive; EB-R-C: elementary branching-rod-coccus growth cycle; H-R-C: hypha-rod-coccus growing cycle

LEGENDS TO THE FIGURES

Figure 1: Microphotographs of strain CR-53 cells obtained by optical (A), scanning (B), and transmission (C) microscopy. Snapping division and septum formation can be observed. The bars within each picture indicate the size proportion.

Figure 2: Phylogenetic tree based on the 16S rDNA sequences of strain CR-53 and its closest *Rhodococcus* type strain species. The bar indicates the 0.5% estimated difference in nucleotide sequences. Numbers on the branches are bootstrap values. The accession numbers of the 16S rDNA sequences used to perform the phylogenetic tree are the following: *R. erythropolis* ATCC 4277^T (X81929), *R. marinonascens* DSM 43752^T (X80617), *R. rhodnii* DSM 43336^T (X80621), *R. ruber* DSM 43338^T (X80625) and *R. rhodochrous* DSM 43241^T (X79288).

Figure 3: Zymogram on MUF-butyrate of 50X concentrated cell extracts (1) and 50X concentrated supernatants (2) from strain CR-53, analysed on SDS-PAGE. The molecular mass of the hypothetical esterase from strain *R. erythropolis* CR-53 is indicated.

CR-53

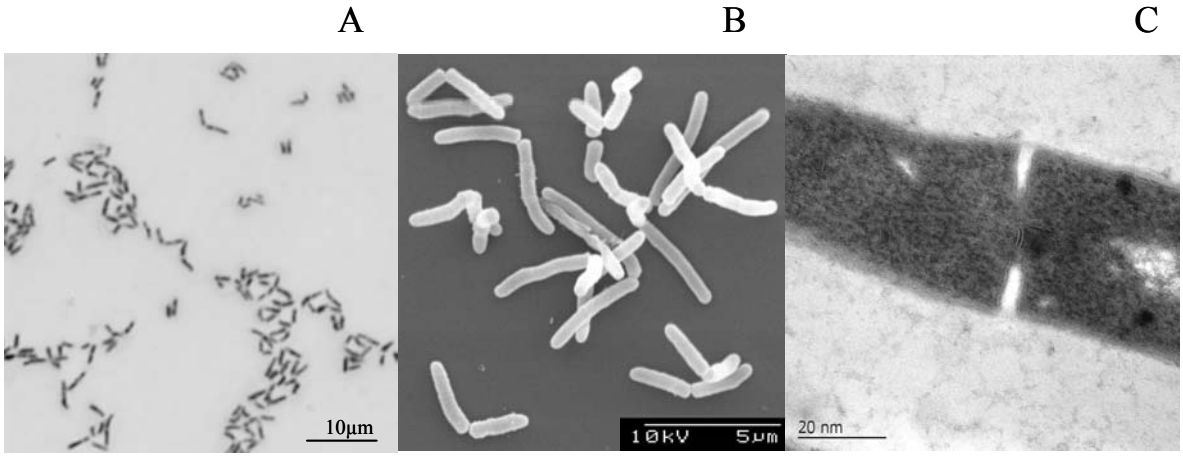

Figure 1: Falcocchio et al

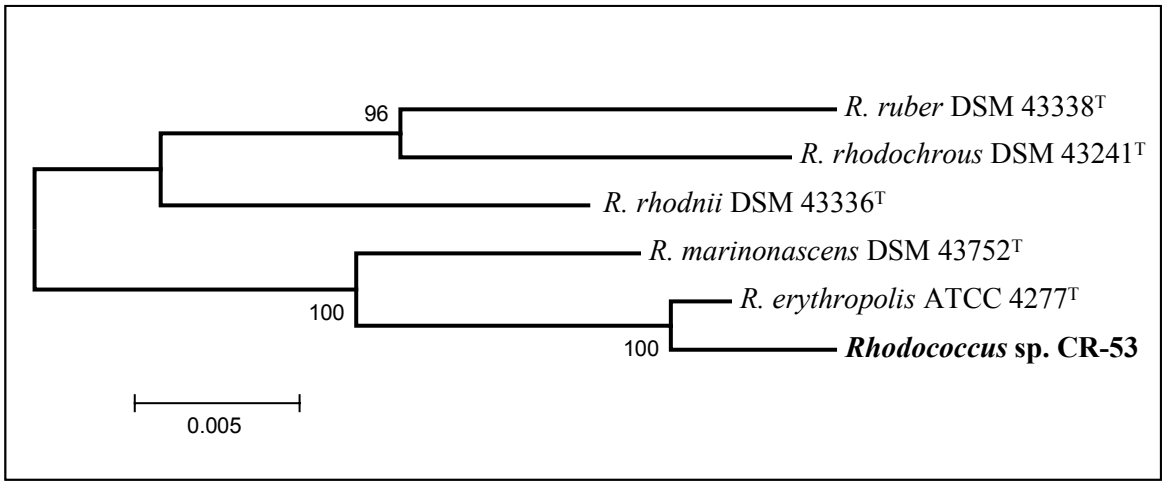

Figure 2: Falcocchio et al

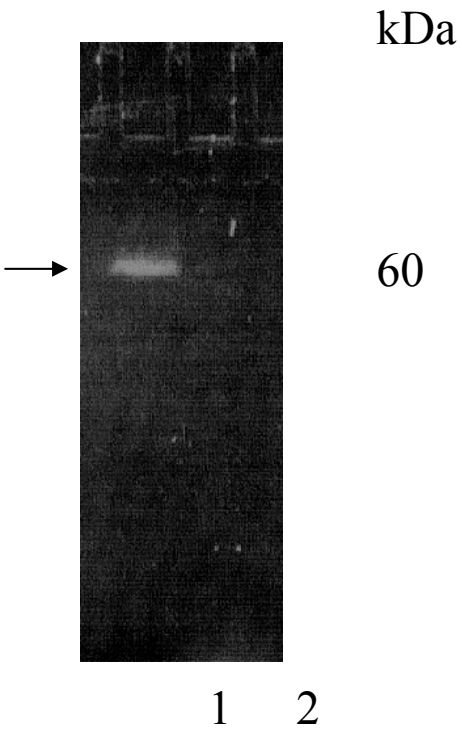

Figure 3: Falcocchio et al.

Inhibition of *Candida rugosa* lipase by saponins, flavonoids and alkaloids

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Running title: Inhibition of *Candida rugosa* lipase by natural substances

Keywords: Candida rugosa lipase, Inhibition, Saponins, Flavonoids, Alkaloids, natural substances.

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ABSTRACT

Lipase inhibitors have generated a great interest because they could help in the prevention or the therapy of lipase-related diseases. Therefore, the aim of the work was to evaluate by HPLC, and using *Candida rugosa* lipase as model, the inhibitory effect of several saponins: β-aescin, digitonin, glycyrrhizic acid (GA) and *Quillaja* saponin; flavonoids: 3-hydroxyflavone, 5-hydroxyflavone, (±)-catechin and kaempferol; and alkaloids: aspidospermine, papaverine, physostigmine, pilocarpine, raubasine, rescinnamine, reserpine and trigonelline.

The inhibition produced by most of these compounds is described here for the first time, and the differences found among them could be related to their structure and physicochemical properties. Saponins appeared very active, being β-aescin and digitonin the most active compounds (IC_{50} = 0.8–2.4 × 10^{-5} M). The inhibitory activity of flavonoids was lower than that of saponins (except GA), and (±)-catechin and kaempferol were the most active. Alkaloids was the most heterogeneous group assayed, varying from rescinnamine, with an IC_{16} similar to that of digitonin, to papaverine and others which showed almost no inhibition.

In conclusion, β-aescin, digitonin, kaempferol or (±)-catechin, strong lipase inhibitors with a low toxicity and present also in antiacne or antiulcer herbal drugs, are promising candidates for the treatment or prevention of lipase-related diseases.

Abbreviations: Crl, *Candida rugosa* lipase; IC, inhibitory concentration; β-NL, β-naphthyl laurate; GA, glycyrrhizic acid; □S, *Quillaja* saponin; HF, hydroxyflavone.

INTRODUCTION

Lipases and esterases are glycerol ester hydrolases (E.C. 3.1.1.–) acting on acylglycerols to liberate fatty acids and glycerol. Several lipases produced by microbial pathogens play an important role in infective diseases. Indeed, *Propionibacterium acnes* lipase and its inhibition by antiacne compounds have been studied because the fatty acids produced by *P. acnes* lipase activity on sebaceous triglycerides induce severe inflammation (Higaki 2003). It has also been described that *Helicobacter pylori* lipase activity can weaken the barrier properties of mucus by hydrolyzing endogenous lipids (Slomiany & Slomiany 1992; Smoot 1997), and it is inhibited by sucralfate and other antiulcer drugs (Slomiany et al 1992; Slomiany et al 1994). Furthermore, lipase-producing fungal dermatophytes can efficiently colonize the keratinized layers of the skin producing cutaneous diseases (Mushin et al 1997). Therefore, research on new lipase inhibitors for the therapy of these diseases and also for other pathologies like obesity, has generated a great interest.

On the other hand, lipolytic enzymes are currently attracting an increasing attention because of their biotechnological potential (Benjamin & Pandey 1998; Jaeger et al 1999). Inhibition studies on lipases could contribute to better understand their mechanism of action in order to design novel substrate specificities for improving the biotechnological applications of these enzymes (Simons et al 1999).

Plant secondary metabolites present in herbal drugs and food have shown to be very useful in the prevention and treatment of many diseases (Singh et al 2003). Among these metabolites, saponins, flavonoids and alkaloids are a promising source of lipase inhibitors since they are present in high concentrations in plant extracts capable of inhibiting porcine pancreatic lipase activity (Shimura et al 1992). Furthermore, these compounds are also present in several plant extracts that have been used for the treatment of diseases in which lipases could play an important role like ulcer (Borrelli & Izzo 2000) and acne (Higaki 2003). Recent studies have also demonstrated that saponins like platycodin D (Han et al 2002), flavonoids like quercetin (Gatto et al 2002), and alkaloids like berberine and sanguinarine (Grippa et al 1999) are good lipase inhibitors. However, further studies are necessary to elucidate the effect of other purified plant

metabolites on lipolytic enzymes in order to select the most suitable ones for therapeutic or preventive pharmacological treatments.

Here, we report the inhibitory effect of several saponins, flavonoids and alkaloids (Fig. 1) on *Candida rugosa* lipase (Crl). This enzyme is well known and widely used in biotechnology (Benjamin & Pandey 1998), thus analysis of its activity and inhibition could help in controlling and increasing its effectiveness in a wide range of biotechnological processes. Moreover, Crl could serve as a model for fungal pathogenic lipases, and its inhibition could be of interest in the treatment against *C. rugosa* strains refractory to antifungal therapy involved in veterinary mycology and in emerging pathogenesis on immunocompromised patients (Colombo et al 2003). For these reasons, Crl has been recently used as model for lipase inhibition assays (Grippa et al 1999; Gatto et al 2002; Ruiz et al 2004).

MATERIALS AND METHODS

Reagents

Methanol, acetonitrile, and water for HPLC were purchased from Lab-Scan (Dublin, Ireland). Trifluoroacetic acid and ethyl acetate (EA) were obtained from Aldrich (Milwaukee, WI, USA). β-naphthyl laurate (β-NL), β-naphtol and kaempferol were purchased from Fluka (Buchs, SG, Switzerland). Raubasine, rescinnamine and aspidospermine were obtained from Simes Spa (Milan, Italy). Digitonin was purchased from ICN Biomedicals Inc. (Irvine, CA, USA). *C. rugosa* lipase (Crl, cat. No. L-1754), β-aescin, glycyrrhizic acid, *Quillaja* saponin, (±)-catechin, 3-hydroxyflavone and 5-hydroxyflavone, reserpine, pilocarpine, physostigmine and the other reagents were from Sigma (St. Louis, MO, USA). All reagents were used without further purification.

Evaluation of lipase inhibition by HPLC

Lipase inhibition assays by HPLC were performed as previously described (Grippa et al 1999). Essentially, the substances under evaluation were dissolved at their maximum solubility in a proper solvent such as water (*Quillaja* saponin, trigonelline, papaverine and pilocarpine), acetone (3-hydroxyflavone and 5-hydroxyflavone), dimethyl sulfoxide (DMSO; glycyrrhizic acid and kaempferol), or methanol (the rest of substances evaluated). Then, 2020 µl reaction mixtures containing varying inhibitor concentrations (from 0 to their maximum solubility), 3.75% inhibitor solvent, 0.46 mM β-NL, 1.25 % acetone, 1 mM sodium taurocholate, 3.5 mM NaCl, 1.5 mM $CaCl_2$, 50 mM Tris–HCl buffer (pH 7.4 at 22°C) and 10 µg ml^{-1} of Crl, were incubated for 30 minutes at 37 °C under gentle mixing. Then, β-naphthol was extracted with 2 ml of ethyl acetate, and 500 µl of the organic phase were withdrawn, evaporated at room temperature under a nitrogen stream and redissolved in 1 ml methanol. Aliquots of 50 µl were analyzed at room temperature using a C-18 reversed-phase column (4.6 × 250 mm; 5 µm particle size, 90 Å pore size; Beckman) equilibrated at a flow rate of 1 ml min^{-1}, with a mobile phase consisting of 40% (v/v) acetonitrile in water, containing 0.1% trifluoroacetic acid. The eluate was monitored at a wavelength of 230 nm with a sensitivity of 0.8 A.U.F.S. The chromatographic system consisted of a precision pump

(Waters, model 515) and a variable wavelength monitor (Waters, model 2487). The area under the chromatographic peak was measured using the Millennium 32 chromatography manager 4.0 software package for Windows®. β-NL unspecific hydrolysis was subtracted performing proper blanks.

Statistical analysis

Lipase inhibition was calculated from the residual activity detected in the presence of the compound under assay with respect to that of untreated samples (without inhibitor but prepared and analyzed under the same conditions than the inhibitor-treated samples, and including the inhibitor solvent to take into consideration the effect of each solvent in Crl activity). The concentrations yielding a lipase inhibition of 16% (IC_{16}) and 50% (IC_{50}) were calculated from the inhibition rate *vs*. inhibitor concentration curves by regression analysis performed using the software Sigma-Plot 8.0 (SPSS, Chicago, IL, USA). Three or more replicates of regression curves with Rsquare coefficients higher than 0.99 were used for IC calculations, being each replicate the result of an independent HPLC assay performed in duplicate.

RESULTS

I. Effect of saponins on *Candida rugosa* lipase

The effect of β-aescin, digitonin, glycyrrhizic acid (GA) and *Quillaja* saponin (□S) on *Candida rugosa* lipase (Crl) was evaluated by HPLC (Table 1; Fig. 2). All saponins showed a high inhibition on Crl, being the most active group of inhibitors in general terms. Both digitonin and β-aescin were the most active compounds (IC_{50} = 0.8–2.4 × 10^{-5} M). β-aescin was the most active inhibitor at concentrations below 2–5 × 10^{-5} M, and digitonin displayed the highest inhibition above this concentration. On the other hand, GA produced the lower inhibition among the saponins evaluated (IC_{16} = 8.5 × 10^{-4} M). □S showed an increasing inhibition at concentrations up to 1.5–2.2 × 10^{-4} M (about the IC_{50}). However, above these concentrations the inhibition by □S remained stable between 50–60 %, in contrast to the other saponins which always produced a higher inhibition when increasing concentrations were assayed (Table 1, Fig. 2).

II. Effect of flavonoids on *Candida rugosa* lipase

Flavonoids produced a lower inhibition than saponins on Crl (except GA) when they were assayed by HPLC. All flavonoids displayed a similar IC_{16} (2.8 × 10^{-4}–1.1 × 10^{-3} M) with (±)-catechin and kaempferol having an IC_{50} of 6.3 × 10^{-3} and 7.5 × 10^{-3} M, respectively. At low concentrations kaempferol was the most active flavonoid, although (±)-catechin was the most efficient at high concentrations. 5-hydroxyflavone (5-HF) showed an intermediate inhibition at low concentrations, halfway between (±)-catechin and kaempferol; however, 5-HF caused an inhibition more than two-fold higher than the isomer 3-hydroxyflavone (3-HF) (Table 1, Fig. 2).

III. Effect of alkaloids on *Candida rugosa* lipase

The inhibition obtained by HPLC assays on Crl by alkaloids was very different among the compounds analyzed. On the one hand, rescinnamine was the most active

alkaloid, displaying an IC_{16} of 2.3×10^{-6} M, similar to those of digitonin and β-aescin. However, this alkaloid was less active than the mentioned saponins at high concentrations ($IC_{50} = 1.6 \times 10^{-4}$ M). Reserpine, the second most active alkaloid, showed an inhibition rate higher than flavonoids, and similar to □S. Aspidospermine displayed an inhibition lower than saponins (except GA) and similar to most of the flavonoids at low concentrations (IC_{16}), but it was more active than flavonoids at high concentrations ($IC_{50} = 1.0 \times 10^{-3}$ M). Trigonelline produced the lowest inhibition among the active compounds, while papaverine, physostigmine, pilocarpine and raubasine produced almost no inhibition (Table 1, Fig. 2).

DISCUSSION

The effect of several saponins, flavonoids and alkaloids on *C. rugosa* lipase (Crl) was analyzed to evaluate their potential as antilipase drugs. Among the compounds tested, saponins were very active, flavonoids displayed a lower inhibition, and alkaloids, the most heterogeneous group, showed a wide inhibition range (Table 1, Fig. 2). The different inhibition produced by these substances is probably related to their different structure (Fig. 1) and physicochemical properties, as explained below.

Saponins

Saponins are glycosidic compounds containing a steroid or triterpenoid sapogenin nucleus. They are characterized by antimicrobial, hypocholesterolemic, anti-inflammatory and other activities and by a low toxicity (Milgate & Roberts 1995; Sirtori 2001; Singh et al 2003). They also have a certain activity against ulcer (Borrelli & Izzo 2000) and human pathogenic *Candida* spp. (Bader et al 2000). *In vitro* inhibition by saponin-containing formulations on *P. acnes* lipase (Higaki et al 2000) and by purified saponins (platycodin D and dioscin) on pancreatic lipases (Han et al 2002; Kwon et al 2003) has been also reported recently. However, saponins from *Medicago sativa* activated pancreatic lipase (Sroka et al 1997).

Here, we have analyzed the effect of *β*-aescin, digitonin, glycyrrhizic acid (GA) and *Quillaja* saponin (□S) on Crl using an HPLC assay (Table 1, Fig. 2). Direct inhibition of a lipase by these saponins is described here for the first time. Only inhibition of the hydrolysis of acetyl fluorescein (substrate of lipases and esterases) by β-aescin on several bacterial strains (Zablotowicz et al 1996), inhibition of a cholesterol esterase by digitonin (Karmanskii 1983), inhibition of pancreatic lipase by GA-containing plant extracts (Shimura et al 1992), and the presence of GA and □S in plant formulations that inhibited *P. acnes* lipase (Higaki et al 2000) have been reported before.

Saponins are known detergents due to their amphiphilic nature and surface activity (Milgate & Roberts 1995). Detergents are considered unspecific reversible lipase inhibitors that possibly interfere with the enzyme, the substrate or the interface (Patkar & Björkling 1994). The higher contents of sugars and their branching could be responsible for the higher inhibition found for β-aescin, digitonin and □S with respect to GA, which bears only a disaccharide. In fact, Crl catalytic triad is in the first part of a narrow tunnel (diameter of 4 Å) (Pleiss et al 1998; Cygler & Schrag 1999) in which the aglicone nucleus of saponins is too big to enter. We think that the aglicone nucleus remains outside of the tunnel, in part at the previous hydrophobic patch (Cygler & Schrag 1999), and only the sugar chains enter in the first part of the tunnel interfering with the catalytic triad of the enzyme. Thus, longer sugar chains would be more able to reach the catalytic triad inside the tunnel than the short ones, which would explain the lower inhibiton produced by GA, the compound with the shorter sugar chains.

Flavonoids

Flavonoids are benzo-γ-pyrone derivatives that can be grouped according to the presence of different substituents and the degree of benzo-γ-pyrone ring saturation. They can also be glycosidated or methylated. Flavonoids are capable of modulating the activity of many enzymes and cell systems producing, among others, antitumoral, cardioprotective or anti-inflammatory effects (Di Carlo et al 1999). They are also components of antiacne formulations, and some of them, like kaempferol, have shown antiulcer activity (izzo et al 1994). Furthermore, some flavonoids like quercetin (Gatto et al 2002) and hesperidin or neohesperidin (Kawaguchi et al 1997) are known lipase inhibitors.

We have evaluated the inhibition produced by (±)-catechin, kaempferol, 5-hydroxyflavone (5-HF) and 3-hydroxyflavone (3-HF) on Crl (Table 1, Fig. 2). All of them displayed a similar IC_{16} and a lower inhibition than saponins (except GA). It has previously been reported that catechin-rich extracts or catechin-related compounds like (–)-epicatechin inhibit pancreatic or gastric lipases and rat adipose tissue–derived lipoprotein lipase, although they do not inhibit hormone-sensitive lipase (Shimura et al 1992; Juhel et al 2000; Yoshikawa et al 2002). Inhibition of pancreatic lipase by

kaempferol, a compound similar to quercetin, or by extracts of *Tea sinensis*, rich in kaempferol, has been reported (Shimura et al 1992; Shimura et al 1994). However, microbial lipase inhibition by (±)-catechin and kaempferol, and lipase inhibition by 5-HF and 3-HF, are described here for the first time.

The mechanism of enzyme inhibition by flavonoids is unclear. The flavonoids analysed are small molecules able to enter in the tunnel of Crl and able to interact with the hydrophobic and hydrophilic residues of the tunnel (Pleiss et al 1998; Cygler & Schrag 1999) because of being aromatic molecules with hydrophilic groups (mainly hydroxyl groups). The similar inhibition observed in this work for (±)-catechin and kaempferol, in comparison with the lower effect produced by 3-HF and 5-HF, mainly at high concentrations, suggests that a larger number of hydroxyl groups is necessary to obtain a higher solubility of these compounds which allows reaching a higher lipase inhibition in the assay. With respect to the conformation of these molecules, the non-planar conformation of catechin seems to favour inhibition of prostaglandin endoperoxide synthase with respect to planar compounds like quercetin and kaempferol (Kalkbrenner et al 1992). However, the similar inhibition produced by (±)-catechin and kaempferol discards this hypothesis and suggests that a pivotal role could be associated to the presence of an ortho-hydrochinon system on B ring. On the contrary, the occurrence of a carbonyl function on the benzopyran moiety seems to be no essential, considering the efficiency observed for (±)-catechin. Moreover, it must be considered that two enantioforms of (±)-catechin are operative, perhaps with different activity grade. The requirement of a polyoxydrylated compound for the inhibition of Crl by the above considered polyphenols is also in agreement with the model proposed for the inhibition of the H^+, K^+-ATPase by polyphenols (Murakami et al 1999).

Alkaloids

Alkaloids are very diverse natural substances (and their related synthetic compounds) that contain nitrogen, usually as a part of a cyclic system. They are active on many enzymes and biological systems, like the central nervous system, producing a wide range of effects (Evans, 1996). Moreover, berberine and other alkaloids are known lipase inhibitors (Grippa et al., 1999).

We have analysed here the effect on Crl of several heterocyclic alkaloids. Inhibition was very different among the compounds assayed, even for those belonging to the same structural group. Among the indole-benzopyrrole alkaloids, rescinnamine was the most active, displaying an IC_{16} similar (but a higher IC_{50}) to those of digitonin and β-aescin. Reserpine was the second most active alkaloid, showing an inhibition higher than flavonoids and similar to □S. Aspidospermine displayed an inhibition rate similar to most flavonoids, and physostigmine and raubasine caused almost no inhibition of Crl. Among the non-indole-benzopyrrole alkaloids, only trigonelline (pyridine and piperidine group) was active, but it caused a low inhibition, and papaverine (isoquinoline group), pilocarpine (imidazole or glyoxaline group) did not inhibit Crl (Table 1, Fig. 2). Lipase inhibition by the mentioned alkaloids is reported here for the first time, except for reserpine and physostigmine. Among other effects, reserpine increased lipoprotein lipase activity of heart tissue (Heinroth & Forster 1980) and inhibited epididymal hormone-sensitive lipase but not that of other tissues (Matsuara, 1970). Physostigmine, a known esterase inhibitor, inhibited also lipases like bile-salt stimulated lipase (Ellis & Hamosh 1992) or pancreatic lipase (Hall 1961), but not lipolytic liver extracts (Katz 1957). Moreover, some isoquinoline-group alkaloids similar to papaverine like palmatine were inactive on Crl, although berberine and others inhibited Crl (Grippa et al 1999).

Inhibition by alkaloids is difficult to explain as they have very different structures. Except trigonelline, most of them have a large structure with several rings which makes difficult their entering in the narrow tunnel of Crl (Pleiss et al 1998; Cygler & Schrag 1999). This fact could explain the lack of inhibition of some of them. Further considerations are possible with respect to the indole-benzopyrrole alkaloids. Raubasine (inactive) and rescinnamine or reserpine (the most active) share the same indole nucleus (Fig. 1: C5,C6,C7). Thus, it is clear that the inhibition produced by rescinnamine and reserpine is, respectively, due to the [(2E)-1-oxo-3-(3,4,5-trimethoxyphenyl)-2-propenyl]oxy or the [(3,4,5-trimethoxybenzoyl)oxy] groups bind to C18 in the indole nucleus of the alkaloid. We think, as we suggested for the sugar chains of saponins, that these two groups can enter into the tunnel and interfere with the active site of the enzyme while the indole nucleus remains outside the tunnel. This

hypothesis is supported by the fact that rescinnamine, which group could enter more deeply than that of reserpine in the tunnel of Crl, is more active than reserpine.

Conclusions and clinical implications

Saponins, (±)-catechin and kaempferol, produced a high Crl inhibition, and some of them have been described to be active against acne, ulcer or pancreatic lipase, as explained before. Moreover, almost all of them have a low toxicity. For example, β-aescin is currently used in the treatment of peripheral vascular diseases as it displays more effectiveness and tolerability than the conventional therapy (Sirtori 2001). At high concentrations, (±)-catechin produces toxic effects like haemolysis, and kaempferol is genotoxic. Nevertheless, they are some of the most occurring compounds among the approximately 1 g of flavonoids that contains the daily human diet (Di carlo 1999). Therefore, these inhibitors are promising candidates for the prevention (creams, shampoos, etc.) and the therapy of diseases in which lipases play an important role like acne, *H. pylori*-associated ulcers, obesity, or fungal diseases. For example, β-aescin, which has also a fungistatic activity (Gruiz 1996), could be very suitable for candidiasis. However, further studies are necessary to confirm their pharmacological potential. On the contrary, the potential use of rescinnamine and reserpine to treat lipase-related diseases is more limited by their other marked pharmacological activities (La Barre, 1960). In conclusion, knowledge about the effect on Crl of these compounds will be helpful with respect to their application in pharmacology, in the designing of new lipase inhibitors, and in the evolution of the active site of the enzyme in order to carry out new biotechnological processes.

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Table 1. Effect of natural substances on *Candida rugosa* lipase evaluated by HPLC assay.

SUBSTANCE	S_{max}[1] (M)	IC_{16} (M)[2]	IC_{50} (M)[2]
I. Saponins			
β-aescin	4.0×10^{-4}	7.5×10^{-7}	8.0×10^{-6}
Digitonin	1.5×10^{-4}	5.9×10^{-6}	2.4×10^{-5}
Glycyrrhizic acid	2.0×10^{-3}	8.5×10^{-4}	$> S_{max}$
***Quillaja* saponin**	1.4×10^{-3}	1.0×10^{-4}	5.5×10^{-4}
II. Flavonoids			
3-hydroxyflavone	1.5×10^{-3}	1.1×10^{-3}	$> S_{max}$
5-hydroxyflavone	2.5×10^{-3}	4.5×10^{-4}	$> S_{max}$
(±)-Catechin	3.0×10^{-2}	8.3×10^{-4}	6.3×10^{-3}
Kaempferol	1.4×10^{-2}	2.8×10^{-4}	7.5×10^{-3}
III. Alkaloids			
Aspidospermine	2.5×10^{-3}	6.3×10^{-4}	1.0×10^{-3}
Papaverine	2.0×10^{-3}	**Inactive**	
Physostigmine	3.8×10^{-2}	**Inactive**	
Pilocarpine	1.0×10^{-2}	**Inactive**	
Raubasine	1.2×10^{-3}	**Inactive**	
Rescinnamine	8.0×10^{-4}	2.3×10^{-6}	1.6×10^{-4}
Reserpine	4.5×10^{-4}	1.1×10^{-4}	4.0×10^{-4}
Trigonelline	5.0×10^{-2}	6.8×10^{-3}	2.0×10^{-2}

[1] Highest concentration at which each substance was tested expressed as mol/l (M).
[2] Inhibitory concentrations (IC) 16% and 50% calculated from the inhibition *vs.* inhibitor concentration curves.

Table 1, Ruiz et al

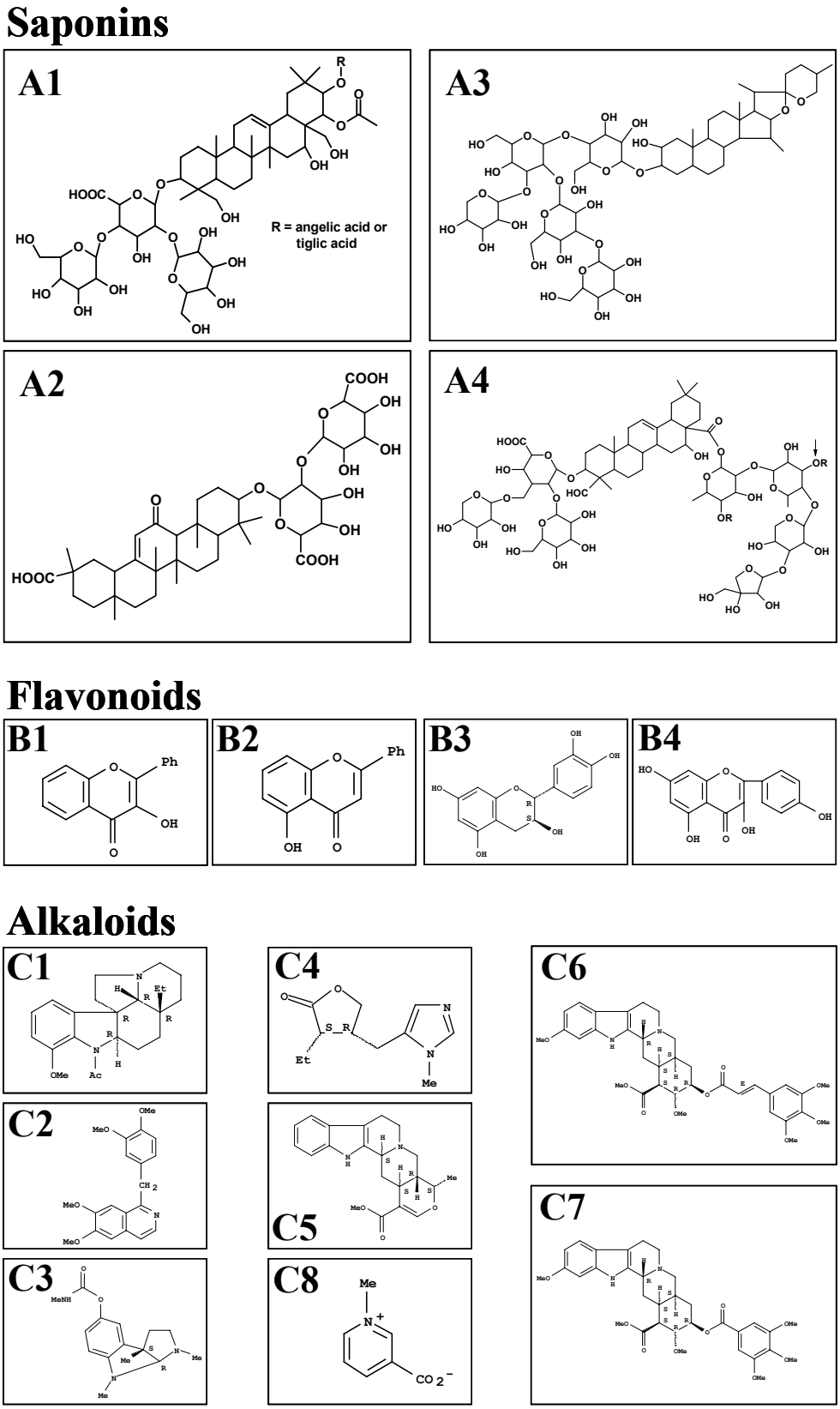

Figure 1. Ruiz et al

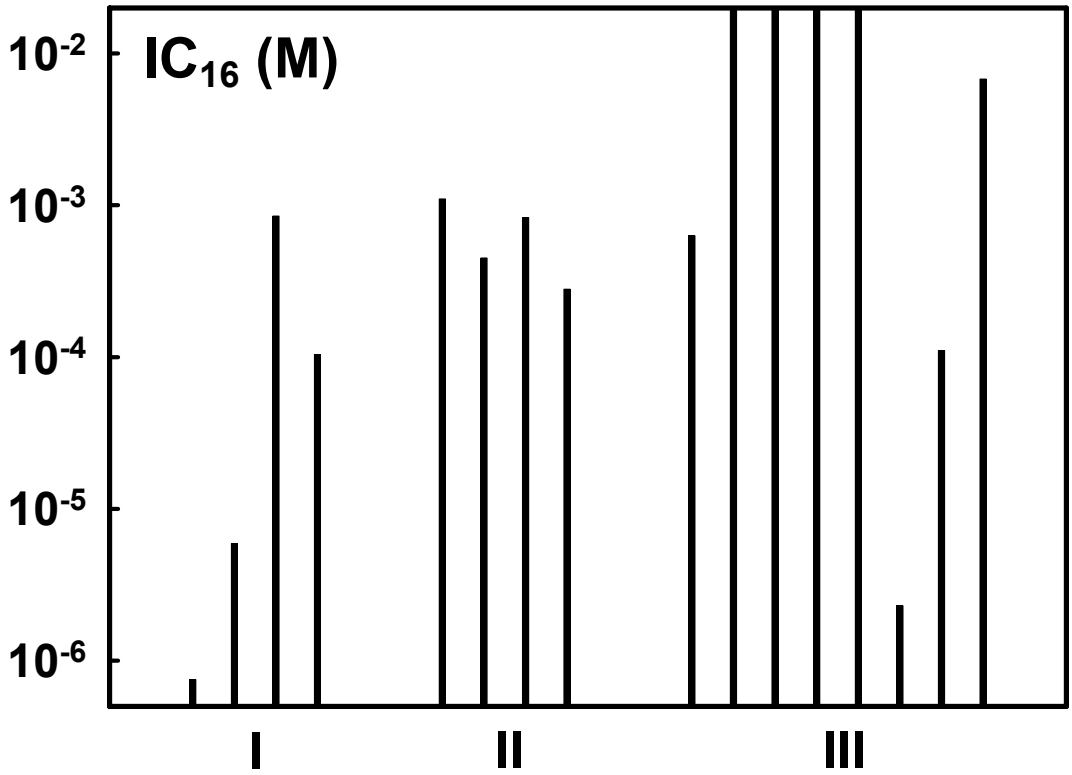

Figure 2. Ruiz et al

LEGEND TO THE FIGURES

Figure 1. Natural substances assayed on *Candida rugosa* lipase. A. SAPONINS: 1. β-Aescin, 2. Glycyrrhizic acid, 3. Digitonin, 4. *Quillaja* saponin; B. FLAVONOIDS: 1. 3-hydroxyflavone, 2. 5-hydroxyflavone, 3. (±)-Catechin 4. Kaempferol; C. ALKALOIDS: 1. Aspidospermine, 2. Papaverine, 3. Physostigmine, 4. Pilocarpine, 5. Raubasine, 6. Rescinnamine, 7. Reserpine, 8. Trigonelline.

Figure 2. Effect of natural substances on *Candida rugosa* lipase evaluated by HPLC. Inhibitory concentrations 16 (IC_{16}) are plotted in the same order as reported in Table 1. I, saponins; II, flavonoids; III, alkaloids.

Helicobacter pylori 26695 ORF HP0739 codes for a previously unknown family V caboxylesterase. Cloning, purification, characterization and inhibition of the enzyme by natural substances

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Running Title: *Helicobacter pylori* carboxylesterase EstV

Keywords: Lipase, esterase, carboxylesterase, dienoate hydrolase, *Helicobacter pylori*

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ABSTRACT

Helicobacter pylori, a Gram-negative pathogen responsible for most infective gastritis and peptic ulcers displays a complex enzymatic and cytotoxic system responsible for the colonization of gastric mucousa. Among the putative virulence factors described for this bacterium, a lipase and a phospholipase seem to play a role in invasiveness by producing the release of fatty acids and other nutrients useful for bacterial cell proliferation. Although the existence of lipase activity has already been described in *H. pylori*, no other information on the genetic, biochemical or structural properties of the responsible enzyme(s) is available. Thus, an exhaustive search for putative lipase-coding genes was performed on the available *H. pylori* 26695 genome. A putative lipase-coding gene (ORF HP0739) was identified, isolated by PCR amplification and cloned in *E. coli*. The amino acid sequence deduced from this ORF showed the consensus sequences and motifs of family V bacterial lipases, although the protein had been annotated as a putative dienoate hydrolase. Purification and subsequent analysis of the gene product demonstrated that it was in fact a 28 kDa carboxylesterase acting mostly on short-chain length substrates, with no interfacial activation, that was designated EstV. Maximum activity of the enzyme was achieved at 50ºC, pH 10 on MUF-butyrate, was stable for at least 30 days when incubated at 4ºC and did not require any additional cofactors. Inhibition studies performed using several natural substances indicated that certain saponins like β-aescin and glycyrrhizic acid are very promissing candidates for the therapy of *H. pylori*-related ulcers.

INTRODUCTION

Helicobacter pylori (formerly *Campylobacter pylori/pyloridis*) is a Gram-negative, spiral-shaped, flagelated, microaerophilic rod mainly found in the antral and fundic segments of the stomach and in the duodenum of humans and other animals. Since it was isolated by Marshall and Warren in 1983 (29), *H. pylori* has generated a great interest because it colonizes about 60% of the world's population, causing gastritis or peptic ulcer disease, and being strongly associated with gastric carcinoma, gastric lymphoma and other weird diseases (15, 34). The mode of transmission of *H. pylori* infection is not well recognized, although it could be related to fecal–oral transmission, direct transmission, or to nosocomial infections (34). Either by direct contact or through elaboration of enzymes and putative cytotoxins, *H. pylori* can directly injure gastric cells and may be responsible for most of the gastric damage found in infected patients. However, *H. pylori* populations are highly diverse (genotypic variation between strains, and variations in populations within an individual host), and this diversity is extremely important in relation to the clinical outcome of infection (http://www.helico.com/; http://www.helicobacterspain.com).

Surface gastric epithelial cell injury is a characteristic feature of gastritis, although there are different opinions on whether most of epithelial damage occurs as a direct result of *H. pylori*-induced cell injury or due to the autoimmune cell damage resulting from the abundant inflammatory response appearing after *H. pylori* infection (54). *In vitro* studies allowed to identify several cytotoxins that may play a role in the pathogenicity of *H. pylori* (58). It is thought that cytotoxins CagA, VacA, IceA, or SabA among others, are the main responsible for cell lysis, directly and through the inflammatory response they produce, whereas enzymes (urease, protease, lipase, phospholipase, etc) would be mainly involved in favouring bacterial colonization of the epithelium, and in the degradation of the proteins and lipids of the tissue matrix and the mucin-lipid barrier that protects the epithelium from the detrimental agents of gastric juice (54, 57).

The existence of *H. pylori*-related lipase and phospholipase activities was first reported by Slomiany and collaborators (52, 53), who also reported their inhibition by

colloidal bismuth subcitrate and sofalcone, two antiulcer agents. Further studies revealed inhibition of these enzymatic activities by sucralfate, ranitidine bismuth citrate, and other antiulcer agents (38, 40, 41, 50, 51), suggesting the importance of these enzymes in ulcer development. Nowadays, it is thought that lipase and phospholipase activities produced by *H. pylori* are involved in hydrolyzing the lipids of gastric mucus, thus reducing its thickness and hydrophobicity. Weakening the mucus barrier properties favours *H. pylori* colonization of the host cell surface, and makes the epithelium more accessible to gastric acid and pepsin. Moreover, lipase and, mainly, phospholipase activities seem to be related to the disruption of the apical membrane of epithelial cells, to haemolysis, and to the generation of lysophospholipids and other cytotoxic and pro-inflammatory lipids detrimental for the mucous gel and epithelium integrity (6, 54, 57).

Nevertheless, knowledge about these enzymes is still low despite the publication of the genome sequence of *H. pylori* strains 26695 (56) and J99 (1). The presence of carboxylesterase activity has been confirmed by employing NMR spectroscopy (31), although the number and biochemical properties of the lipase(s) produced by this bacterium remains still unknown. *H. pylori* possesses also different phospholipase activities (A_1, A_2, C; 39), although phospholipase A_1 activity is almost unknown, and phospholipase C has been only partially purified and preliminarily characterized (59). Phospholipase A_2 is the only enzyme acting on lipids whose gene (*pld*A) has been identified, cloned and expressed (13). Experiments with isogenic mutants revealed that this enzyme was responsible for more than 90% of the phospholipase A_2 activity, and for 50% of the haemolytic activity of the bacterium. PldA is not essential for adhesion, although *pld*A mutants failed to colonize the gastric mucosa, probably because of an inability to break down the mucous layer to allow penetration and a stable colonization (13, 61). Furthermore, phase variations in *pld*A gene are involved in the adaptation of *H. pylori* to the acidic environment of the stomach lining by changing bacterial surface-lipid composition, which leads to an increased adherence to epithelial cells, and to a higher release of urease, VacA, and other factors involved in colonization, persistence, and epithelium damage (55). On the other hand, *H. pylori* can indirectly induce the release of phospholipase A_2 from the cytosol of the stomach epithelial cells, from Paneth cells of the intestinal mucose, and from polymorphonuclear leucocytes. Current evidences suggest that these enzymes would be the main responsible for the mucus and

epithelium damage, and for the increased production of eicosanoids and other pro-inflammatory and cellular signalling factors associated to phospholipase A_2 activity (21, 35, 42).

H. pylori is thus a significant problem in the world, and its importance is unlikely to diminish in the foreseable future. Therefore, an extensive research is being carried out to understand the pathogenic mechanisms of this bacterium, and to its prevention or eradication. In this work we report the search for lipase-coding genes in *H. pylori* genome, the identification of an ORF coding for a lipase, and the purification, characterization and inhibition of EstV, the gene product of *H. pylori* ORF HP0739.

MATERIALS AND METHODS

Strains, plasmids and growth conditions

Virulent wild-type *Helicobacter pylori* 26695 strain (56) was kindly provided by Dr. R. Araujo. Fresh cultures of the strain grown on blood agar base supplemented with 7% lysed defibrinated horse blood and incubated for 4 days at 37 ºC in a microaerobic atmosphere, were provided each time. *Escherichia coli* strains DH5α (19; *supE44 ΔlacU169* (*ϕ80dlacZΔM15*) *hsdR17*(r_k^- m_k^+) *recA1 endA1 gyrA96 thi-1 relA1*), and XL1-Blue (9; *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F'*proAB lacI*^{q}*ZΔM15* Tn*10* (Tet^r)] were routinely cultured O/N at 37 ºC on Luria-Bertani broth or agar plates, and used as host strains for cloning and expression of lipase-coding genes. Plasmids pBR322 (7) and pUC19 (62) were used as cloning and expression vectors.

Identification of ORF HP0739 in *H. pylori* genome

The amino acid sequences of previously described lipases (3) were confronted through BLAST (2; http://www.ncbi.nlm.nih.gov/blast) to the complete genome sequence of *H. pylori* 26695 (56). Several candidate proteins were found, that were re-analyzed by a second BLAST search performed against all kind of other previously described enzymes. Among them, ORF HP0739 was the only candidate that showed higher similarity to lipases than to other enzymes and displayed the typical motifs of carboxylesterases.

Isolation and cloning of ORF HP0739

Specific primers HPESTFW (5'-**GAA** TAA CAT *ATG* GCC AAA CGC AG-3'; *Nde*I site underlined, fragment of Shine-Dalgarno region in bold, start codon in italics) and HPESTBW (5'-CGC TAA ATG GAT CCA A**CT** **A**AG AC-3'; *Bam*HI site underlined, stop−complementary codon in bold) were designed for PCR amplification

of ORF HP0739, using *Pfu* polymerase (Stratagene) and 29 cycling periods of 30" at 94ºC, 20" at 48 ºC, and 4' at 72ºC. Amplified DNA was purified through WIZARD® columns (Promega), ligated to *Sma*I-digested pUC19, and the resulting recombinant plasmid pUC-EstV was transformed into *E. coli* DH5α to obtain recombinant clone *E. coli* DH5α–pUC-EstV.

Sequence analysis

Both DNA strands of purified PCR products or recombinant plasmids were sequenced using a *BigDye*® Terminator V3.1 Cycle Sequencing Kit (Beckman-Coulter) and the analytical system CE□™8000 (Beckman-Coulter) available at the Serveis Científico Tècnics of the University of Barcelona (45).

Sequence identity was analysed through BLAST (2), alignments were performed using ClustalW Multalign program (20; http://www.ebi.ac.uk/clustalW), and signal peptide identification was performed through SignalP V2.0 software (36). The BioEdit Sequence Alignment Editor (18) was used for several analyses of the DNA sequences obtained, including restriction pattern determination, or identification of open reading frames (ORFs). The physico-chemical parameters and the presence of defined protein regions (ScanProsite: http://www.expasy.org/prosite) were analysed at ExPASY (www.expasy.org). Superfamily assignments and analysis of the structural domains of the proteins were performed using Prodom (48; http://prodes.toulouse.inra.fr/prodom/current/html/home.php) and Pfam (4; http://www.sanger.ac.uk/Software/Pfam/search.html). Prediction of transmembrane regions was analyzed with DAS–Transmembrane prediction server (11; http://www.sbc.su.se/~miklos/DAS/), and prediction of protein subcellular localization was performed using PSORTb v.2.0 for bacterial sequences (16; http://www.psort.org/). Secondary structure prediction was performed using the PSIPRED protein structure prediction server (30; http://bioinf.cs.ucl.ac.uk/psipred/), whereas protein fold recognition using 1D and 3D sequence profiles coupled with secondary structure information was obtained from 3D-PSSM web server (Kelley *et al*., 2000; http://www.sbg.bio.ic.ac.uk/~3dpssm/).

Purification of ORF HP0739 gene product

Presence of lipase activity in *E. coli* recombinant clones bearing ORF HP0739 was confirmed using a fluorescent assay based on MUF-derivative substrates (12, 43) and by plate assays on tributyrin-supplemented medium. The putative lipase coded by ORF HP0739 was purified through FPLC chromatography (AKTA FPLC, Amersham Biosciences), using *E. coli* DH5α/pUC-EstV concentrated cell extracts and a three step gel-filtration/ionic exchange/gel-filtration procedure. Briefly, 2ml cleared cell extract samples were loaded onto a Superdex 200 prep grade column (dextran-agarose matrix; 124 ml; 34 μm mean particle size, Amersham Biosciences) equilibrated at a flow rate of 1 ml min^{-1} with a mobile phase consisting in 50 mM phosphate buffer (pH 7.0) containing 0.02% (w/v) sodium azide. Protein separation was monitored at 280 nm and the eluted protein fractions were analyzed by SDS-PAGE and zymogram (12). Fractions showing maximum lipase activity were recovered and concentrated before being injected into a Mono □ 5/50 GL column (polystyrene/divinyl benzene matrix; 5 ml; 10 μm particle size; 2−12 pH stability; Amersham Biosciences) equilibrated at a flow rate of 2 ml min^{-1} with a mobile phase consisting on 50 mM glycine−NaOH buffer (pH 10; 1 point over the predicted EstV *p*I to have an electrically negative-charged protein able to bind to the column), containing 0.02% (w/v) sodium azide. Protein separation by ionic exchange was performed using a 0−1 M NaCl gradient provided by mixing 50 mM glycine−NaOH buffer (pH 10) containing 0.02% (w/v) sodium azide, with a second buffer contatining 50 mM glycine−NaOH buffer (pH 10), 1M NaCl, and 0.02% (w/v) sodium azide. The eluted protein fractions were analysed for lipase activity and 0.5 ml aliquots of the most active protein fractions were re-purified afer injection into a Superdex 200 10/300 GL column (dextran-agarose matrix; 24 ml; 13 μm mean particle size, Amersham Biosciences) equilibrated at a flow rate of 0.5 ml min^{-1} with a mobile phase consisting in 50 mM phosphate buffer (pH 7.0) containing 0.02% (w/v) sodium azide. The eluted protein fractions containing the desired carboxylesterase were detected by colorimetric activity assay (46) and SDS-PAGE analysis followed by zymogram, and recovered.

Activity assays

Lipolytic activity was detected on agar plates supplemented with 1% tributyrin, olive oil or triolein, and 0.0002% Rhodamine B (25), or by 4-methylumbelliferone (MUF) release from MUF-derivative substrates (12). Activity determination and zymogram assays were routinely performed using crude cell extracts or concentrated culture supernatants, prepared as described before (45). The release of *para*-nitrophenol (*p*NP) or 4-methylumbelliferone (MUF) from *p*NP or MUF-derivative substrates was measured as described (44, 46). 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 were performed as previously described (43, 44). After protein separation, activity was detected by zymogram, and gels were subsequently stained with Coomassie Brilliant Blue R®-250 for protein band visualization (12).

Inhibition assays

Lipase inhibition or activation experiments were performed according to a previously described colorimetric microassay, using *p*NP-laurate as a substrate (46). Lipase inhibition or activation was calculated from the residual activity detected in the presence of the compound under assay with respect to that of untreated samples (without inhibitor but prepared and analyzed under the same conditions than the inhibitor-treated samples, and including the inhibitor's solvent to take into consideration the effect of each solvent in lipase activity). The concentrations yielding a lipase inhibition of 16% (IC_{16}) and 50% (IC_{50}) were calculated from the inhibition rate *vs*. inhibitor concentration curves by regression analysis performed using the software Sigma-Plot 8.0 (SPSS). Three or more replicates of regression curves with Rsquare coefficients higher than 0.99 were used for IC calculations, being each replicate the result of an independent assay performed in duplicate.

RESULTS

Identification of HP0739

Since the only ORF annotated as a lipase-like protein in *H. pylori* genome did not show any similarity to known lipases and did not display the structural features of these enzymes (it was in fact a membrane transporter), the complete genome and proteome of strain *H. pylori* 26695 were analyzed in order to find other ORFs showing similarity to lipases.

For this purpose, the amino acid sequences of previously described bacterial lipases (3, 22) were analyzed through BLAST search against the putative proteins of *H. pylori* 26695. From this analysis, ORFs HP0289, HP0346, HP0739, HP0906 and Omp26, as well as the previously cloned and characterized PldA phospholipase A1, appeared as the putative proteins with the highest similarity to lipases. These candidate proteins were re-analyzed through BLAST search against all kind of other enzymes, being HP0739 the only protein that displayed higher similarity to lipases than to other enzymes and contained the typical motifs of these enzymes.

Sequence analysis

Analysis of *H. pylori* 26695 gene *HP0739* revealed an ORF of 726 bp (from position 794021 to 794746, both included), with the first nucleotide and the last 15 nucleotides overlapping with the previous and subsequent ORFs. Two promoter sequences were found in the upstream region of the gene: region 793809–793859 (score 0.99) and region 793909–793958 (score 1). A sequence displaying the typical features of a ribosome binding site (aagaa) starting 10 bp upstream the start codon, and several inverted repeats located 112 bp downstream the stop codon, that could act as transcription terminators, were also found.

Protein HP0739 (AED07785), annotated as a putative 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase (26.7% BLASTp identity to the epoxide dienoate hydrolase from *Pseudomonas putida* CE2010) by Tomb and collaborators (56), showed the highest

identity (BLASTp) to the hypothetical protein Jhp0676 from *H. pylori* J99 (97% identity) and to other putative proteins from related strains such as *Campylobacter jejuni*. HP0739 showed also a moderate identity (25−40%) to several known and putative lipases from family V (3) and other lipases and hydrolases, whereas its identity to known dienoate hydrolases was lower than 27%. When ClustalW analyses were performed using non-putative proteins, those showing the highest identity (13−21%) and similarity (35−50%) were the bacterial lipases from family V, together with several bacterial lipases from other families such as *Mycoplasma hyopneumoniae* lipase, related to lipases from family II (47), and the family VI carboxylesterase from *Spirulina platensis* (3). On the contrary, the dienoate hydrolases from *Pseudomonas putida* CE2010 and other bacterial strains displayed a much lower identity to HP0739. For this reason, and due to the fact that HP0739 displayed the typical features of family V lipases, the identified protein was preliminarily assigned as a member of family V of bacterial lipases, being named EstV.

Analysis of the predicted EstV protein revealed that it was a protein of 241 amino acids, with a deduced MW of 27527 Da and a theoretical pI of 9.0, without any sequence displaying the features of a signal peptide. EstV showed also a high content in short non-polar residues (35.7% of the total) as is described for enzymes acting on hydrophobic substrates (14). Analysis of the primary structure of this protein revealed also that EstV showed the pentapeptide Gly-His-Ser-Phe-Gly, corresponding to the typical Gly(Ala)-Xaa-Ser-Xaa-Gly pentapeptide of lipases (14).

More detailed computational analysis of the amino acid sequence of EstV revealed that it was a globular, compact and single-domain protein, belonging to the group of serine-hydrolases and displaying the typical α/β hydrolase fold of lipases (23). However, a three-dimensional model of this enzyme could not be obtained due to its low similarity to lipases of known structure. The analysis of the secondary structure predicted for EstV (Figure 1) revealed the presence of 8 β strands, in agreement with the 8 β strands that constitute the typical β sheet of lipases (23), and 7 α helices (Figure 1). This analysis also revealed that the conserved Gly-His-Ser-Phe-Gly pentapeptide of family V lipases, containing the catalytic serine residue (Ser^{99}), forms a turn between strand β5 and the following α helix (Figure 1), corresponding to the most conserved structural arrangement of the α/β hydrolase fold, the so called “nucleophile elbow”,

present in all known lipases (23). Asp^{192} (located in a turn after strand β7) and His^{219} (located after β7) were assigned as the two other members of the catalytic triad of this lipase (Figure 1), according to their position with respect to the prototypic α/β hydrolase fold (23), and on the basis of sequence alignments with other family V lipases. In view of the results obtained from sequence alignments and from molecular and structural analyses, the putative lipase EstV was subsequently cloned and characterized.

Cloning and purification of *H. pylori* EstV

PCR amplification of *HP0379,* including a few nucleotides of the upstream and downstream regions of this gene, was performed as described, using primers HPESTFW and HPESTBW. The resulting 750 bp-amplified DNA fragment was ligated to *Sma*I-digested pUC19 and cloned into *E. coli* DH5α. The recombinant clone obtained (*E. coli* DH5α–pUC-EstV) was analysed on lipid-supplemented CeNAN agar plates to detect the putative lipolytic activity of EstV. Clear hydrolysis zones were observed using tributyrin as a substrate (Figure 2A), whereas no activity was found on plates containing olive oil or triolein (not shown). Moreover, crude cell extracts from the recombinant clone displayed activity on MUF-butyrate and MUF-oleate when assayed by fluorimetric liquid assay (1.7 ± 0.1 and $3.2 \cdot 10^{-2} \pm 0.2 \cdot 10^{-2}$ mU mg^{-1} protein, respectively, after subtraction of the basal activity of the host strain). Maximum lipolytic activity was found in crude cell extracts from cultures induced with 1 mM IPTG at $A_{600\ nm}$ of 0.5−0.6, and subsequently incubated overnight. Purification of EstV was achieved after a three-step FPLC chromatography of crude cells extracts obtained from recombinant clone *E. coli* DH5α–pUC-EstV, as described in Materials and Methods. This process allowed purification of EstV almost to homogeneity (215-fold purified, with a yield of 19.5% and with a specific activity of 664.1 mU mg^{-1} protein). Purified EstV migrated as a band of ca. 28 kDa on SDS-PAGE gels, and displayed low but detectable lipolytic activity on zymograms (Figure 2B).

Enzyme characterization

Purified EstV was tested for lipase activity on several *p*-NP- and MUF-derivatives (Table 1). EstV displayed the typical behaviour of carboxylesterases since it showed preference for short acyl chains. The highest activity (100%) was found on *p*-NP acetate (537.3 ± 26.9 mU mg^{-1} EstV at 55 ºC and pH 7) and on MUF-butyrate (667.9 ± 15.3 mU mg^{-1} EstV at 50 ºC and pH 10). EstV also efficiently hydrolyzed $C_{4–5}$ *p*-NP-derivatives (residual activity higher than 70%), whereas the activity decreased dramatically (residual activity lower than 20%) on *p*-NP- or MUF-derivatives with acyl chains of 8 or more carbon atoms (Table 1).

The effect of temperature and pH on the activity of EstV was determined using MUF-butyrate and *p*-NP butyrate as substrates. The highest activity was found at 50 ºC and pH 10 using MUF-butyrate as a substrate. Moreover, EstV displayed an activity higher than 80% from 45 ºC to 55 ºC, and over 90% at pH 6 (succinate-NaOH buffer) and at pH 9−9.5 (Tris-HCl), whereas the residual activity was 61.2% at pH 7 (phosphate buffer). When *p*-NP butyrate was used as a substrate, the assays were performed at pH 7 to avoid the autohydrolysis of this substrate at pH 10. Interestingly, the optimum temperature was 55 ºC under these conditions, whereas the residual activity was higher than 60% from 34 ºC to 60 ºC. The enzyme remained active for at least 30 days when it was stored at 4 ºC and pH 7 or pH 10.

When the kinetics of EstV was analyzed on MUF-butyrate, the enzyme showed a typical Michaelis-Menten behaviour with no interfacial activation, as described for most carboxylesterases (23). A plot with the maximum activity over $1.2 \cdot 10^{-3}$ M, but showing a decreasing activity at higher concentrations was obtained (not shown). The enzyme displayed a calculated apparent V_{max} and K_M of 695.3 ± 13 mU mg^{-1} and $5 \cdot 10^{-4}$ ± $0.1 \cdot 10^{-4}$ M, respectively, with a k_{cat} of 3.2 ± 0.1 s^{-1} and a k_{cat} / K_M coefficient of $6.4 \cdot 10^{3}$ ± $0.1 \cdot 10^{3}$ s^{-1} M^{-1}.

Inhibition assays

The effect of different agents on the activity of EstV was determined at 55 ºC and pH 7, using *p*-NP laurate as a substrate (Table 2). Among the cations analyzed, Ag^{+}

inhibited almost completely EstV at 1mM. Hg^{2+} produced also a high inhibition (almost 50%) of this enzyme, whereas Cu^{2+} Pb^{2+} and Zn^{2+} caused a significant inhibition of EstV at this concentration (74−86% residual activity). On the contrary, Ba^{2+}, Ca^{2+} and Mg^{2} activated EstV at 1 mM (residual activity higher than 130%). When cations were assayed at 10 mM, no activity was detected for EstV in the presence of Ag^{+}, whereas Cu^{2+} Pb^{2+} and Zn^{2+} caused a high inhibition of this enzyme (residual activity lower that 50%). Co^{2+} and Fe^{2+} produced also a moderate inhibition of EstV at this concentration. On the contrary, Ba^{2+}, Ca^{2+} and Mg^{2+} strongly activated EstV at 10 mM (127%, 150% and 135% residual activity, respectively).

The influence of the amino acid-modifying agents NAI (*N*-acetylimidazole; tyrosine), PHMB (*p*-hydroxymercuribenzoic acid; cysteine) and PMSF (phenylmethylsulfonyl fluoride; serine), and the effect of EDTA, urea and SDS were also tested. PMSF strongly inhibited EstV (41% residual activity at 10 mM), whereas NAI and PHMB caused no significant reduction or even a slight activation of EstV, suggesting that serine, but not cysteine nor tyrosine are involved in the functional or structural domains of the enzyme. Among the other agents assayed, SDS produced the strongest inhibition at both 1 mM and 10 mM (11 and 7% residual activity, respectively). Urea caused also a high inhibition of EstV, whereas EDTA produced a lower inhibition of this enzyme (Table 2).

Inhibition of EstV by several plant secondary metabolites that could help in the therapy of some pathogen-related diseases was also tested (Figure 3). EstV was strongly inhibited by reserpine (IC_{50} = $4.5 \cdot 10^{-5}$ M), whereas β-aescin and glycyrrhizic acid produced a lower inhibition of this enzyme (IC_{16} = $2.9–4.1 \cdot 10^{-4}$ M). Interestingly, (±)-catechin and kaempferol produced a moderate (37.2%) and a high (224.3%) activation of EstV, respectively, when assayed at their maximum solubility concentration. On the contrary, digitonin, *Quillaja* saponin and rescinnamine caused almost no effect on EstV activity.

DISCUSSION

Several lipases produced by microbial pathogens play an important role in infective diseases, being *P. acnes* lipase (GehA; 33) and *H. pylori* lipase(s) among the most interesting from a clinical point of view. *H. pylori* lipase activity has been related to peptic ulcer since it can hydrolyze the endogenous lipids of the stomach and duodenum lining, weakening the barrier properties of mucus, disrupting epithelial cells and generating cytotoxic and pro-inflammatory lipids (57). However, the number and biochemical properties of the lipase(s) produced by this bacterium remain almost unknown, since only related enzymes such as *H. pylori* phospholipases A_2 and C have been cloned and/or characterized (13, 59). Thus, we analyzed the genome and deduced proteome of *H. pylori* 26695 in order to select those putative ORFs that could be responsible for the lipolytic activity of this microorganism. ORF HP0739, showing higher similarity to lipases than to other enzymes and displying the typical lipase motifs, was selected for subsequent cloning and characterization.

HP0739, putatively annotated as a 2-hydroxy-6-oxohepta-2,4-dienoate hydrolase (56), showed the highest identity to putative proteins with no assigned function from related strains such as Jhp0676 from *H. pylori* J99. Among the non-putative proteins, those showing the highest identity to HP0739 were the bacterial lipases from family V (3) and the lipases from *Mycoplasma hyopneumoniae* (related to family II; 47) and *Spirulina platensis* (family VI; 3), whereas its similarity to *Ps. putida* dienoate hydrolase, an enzyme involved in the degradation pathway of aromatic compounds such as toluene (37), and similar enzymes was lower. In fact, it is known that lipases from family V share significant amino acid sequence similarity to various bacterial non-lipolytic enzymes such as epoxide hydrolases, dehalogenases and haloperoxidases, which also possess the typical α/β hydrolase fold and the catalytic triad (3).

Analysis of the amino acid sequence of HP0739 showed that it was a protein of 241 amino acids, with a deduced MW and pI of 27.5 kDa and 9.0, respectively, with no signal peptide. Computational analysis revealed a globular, compact, single-domain protein belonging to the serine-hydrolases group, and displaying the common features of lipases: (*i*) elevated content in short non-polar residues, (*ii*) a typical α/β hydrolase fold constituted by 7 α helices and a β sheet made up of 8 β strands, (*iii*) a catalytic triad

that involves the residues Ser^{99}, Asp^{192} and His^{219}, located at their prototypic positions of the α/β hydrolase fold, and (*iv*) a typical "nucleophile elbow" constituted by the GHSPG pentapeptide that contains the catalytic serine, arranged in a turn between strand β5 and the following α helix (23, 14).

HP0739 was cloned and purified in order confirm its lipolytic activity, and to perform the biochemical characterization of the enzyme. As described for most family V lipases (3), the protein displayed the typical behaviour of carboxylesterases, being active on tributyrin but not on triolein or olive oil, and showing a marked preference for short-chain *p*-NP- and MUF-derivatives. Analysis of the kinetic behaviour of this enzyme on MUF-butyrate revealed a Michaelis-Menten behaviour with absence of interfacial activation, as described for carboxylesterases (23). According to the sequence similarity and the kinetics properties shown, the isolated protein was assigned to family V and designated EstV. Maximum activity of EstV was achieved at 50 ºC and pH 10, displaying also a high activity from 45 ºC to 60 ºC, and at pH 6 and 9−9.5. Moreover, EstV displayed moderate activity at 37 ºC and pH 7. Such optima conditions could seem surprising for an enzyme from an acid-tolerant neutrophile bacterium adapted to a host whose temperature is 37 ºC. Nevertheless, these results are similar to those obtained by Weitkamp and collaborators (59) for the phospholipase C activity of *H. pylori* (maximum activity at 56 ºC and pH 8). Furthermore, it should be considered that the intracellular pH of *H. pylori* displays a high variability in response to pH changes of the environment (e.g. at pH 7.4 the intrabacterial pH is 8.1, although it can rise in the presence of urea; 60).

The effect of different agents on the activity of EstV, showed a significant inhibition by Ag^{+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Pb^{2+} and Zn^{2+} and a certain activation by Ba^{2+}, Ca^{2+} and Mg^{2}, in agreement with the general effects of these compounds on other lipases (17). PMSF strongly inhibited EstV, whereas NAI and PHMB caused no significant reduction or even activation of EstV, suggesting that serine, but not cysteine nor tyrosine are involved in activity. Among the other agents assayed, EDTA produced a low inhibition of this enzyme, whereas SDS and urea produced a very strong inhibition of EstV, which could explain the low activity of EstV on zymograms performed after SDS-PAGE or IEF gels.

H. pylori EstV was strongly inhibited by reserpine, moderately inhibited by β-aescin and glycyrrhizic acid, and was activated by (±)-catechin and kaempferol, whereas the other substances analyzed had no effect on activity. Reserpine has no potential use in the treatment of ulcer since it is ulcerogenic (26). With respect to flavonoids, the activation produced by (±)-catechin and kaempferol indicate that their antiulcerogenic activity, previously attributed to gastroprotective and anti-inflammatory effects, to inhibition of *H. pylori* growth, etc (5), is not related to EstV activity. However, it could be also possible that an increased activity of EstV was detrimental for *H. pylori*. On the contrary, β-aescin and glycyrrhizic acid are very interesting candidates for the therapy of *H. pylori* related-ulcers because, appart from inhibiting *H. pylori* EstV, they display several antiulcer effects. For example, β-aescin produces inhibition of gastric acid and pepsinogen secretion (27), together with an improvement of blood flow (28), whereas glycyrrhizic acid or the corresponding sodium and potassium salts, produced by the intestinal microflora, have gastro-protective effects, producing inhibition of both, *H. pylori* growth (24) and N-actetyl transferase activity (10). Moreover, carbenoxolone, a derivative from glycyrrhizic acid, has been shown to accelerate peptic ulcer healing, probably by enhancing the rate of incorporation of various sugars into gastric mucosal glycoproteins, thus stimulating gastric mucus production, and probably by promoting mucosal cell proliferation (8). In addition, these compounds have a low toxicity and are currently used as therapeutic agents. In this sense, β-aescin is used for the treatment of peripheral vascular diseases, and glycyrrhizic acid is a component of certain herbal drugs used in the therapy of acne, ulcer, and other diseases (32, 49).

The results obtained so far in the biochemical characterization of EstV suggest that this enzyme would not be the main responsible for the lipolytic activity of *H. pylori* described previously (52). In fact, both results are difficult to compare since the assays were performed under very different conditions and using different strains. On the one hand, lipase activity reported by these authors was attributed to an extracellular lipase, whereas EstV did not show the presence of a signal peptide. However, it should be considered that Slomiany and collaborators (52), used as enzyme source the filtrate of a saline solution used to wash plates containing *H. pylori* colonies isolated from different patients, a method that can break the cells and release intracellular proteins. Moreover,

it should also be considered that EstV could bear a signal peptide not detected by the software we used, or could be secreted by a mechanism independent of a signal peptide, like the ABC-transport system used by some Gram-negative lipases lacking a typical N-terminal signal sequence (22). On the other hand, the optimum temperature and pH of 37 ºC and pH 7.2 reported by these authors significantly differs from those we obtained, although EstV displayed also high activity under these conditions. It should also be taken into account that the assays performed by these authors were carried out using thin layer-chromatography and ^{3}H-labeled triolein as a substrate, in a temperature range of 20−45 ºC and in a pH range of 6−8. Moreover, these authors did not report any data about the substrate preferences and enzyme kinetics of the lipase activity of their filtrates, which did not allow a complete comparison with the results obtained in this work. Therefore, it seems that the lipase activity detected by these authors could mainly correspond to another of the lipase candidates found in the screening of *H. pylori* proteome, or to a different protein not selected during our analysis. On the contrary, EstV could be responsible for the *H. pylori* carboxylesterase activity previously detected (31) using ^{19}F NMR spectroscopy to analyze the hydrolysis of ethyl fluoroacetate and dimethyl fluoromalonate produced by *H. pylori* cell suspensions. In fact, these authors obtained the maximum activity on ethyl fluoroacetate, which would be in agreement with the maximum activity on *p*-NP acetate obtained for EstV in this work. Anyhow, further analysis will be performed to investigate the physiological and pathogenic significance of EstV *in vivo* and *in vitro*.

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TABLE 1. Substrate profile of EstV

Substrate	EstV Activity	
	mU mg^{-1} EstV	**%**
p-NP acetate ($C_{2:0}$)	537.3	100.0
p-NP butyrate ($C_{4:0}$)	478.2	89.0
p-NP valerate ($C_{5:0}$)	396.6	73.8
p-NP caproate ($C_{6:0}$)	242.5	45.1
p-NP caprylate ($C_{8:0}$)	102.5	19.1
p-NP caprate ($C_{10:0}$)	62.9	11.7
p-NP laurate ($C_{12:0}$)	54.3	10.1
p-NP palmitate ($C_{16:0}$)	2.2	0.4
p-NP stearate ($C_{18:0}$)	0.6	0.1
MUF-butyrate ($C_{4:0}$)	667.9	100.0
MUF-oleate ($C_{18:1c\Delta9}$)	71.5	10.7

The standard deviations obtained ranged from 2% to 10% of the corresponding mean values.

All the substrates were assayed at a concentration of 1 mM. MUF-derivatives were assayed at 50 ºC and pH 10, the optima reaction conditions of EstV, whereas *p*-NP-derivatives were assayed at pH 7 and 55 ºC (the optimum temperature of EstV at this pH).

Table 1. Ruiz et al

Figure 1: Ruiz et al.

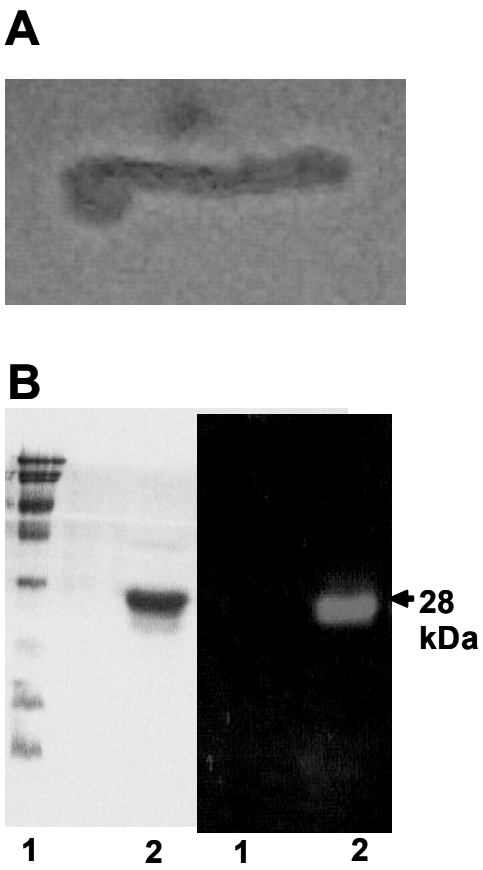

Figure 2: Ruiz et al.

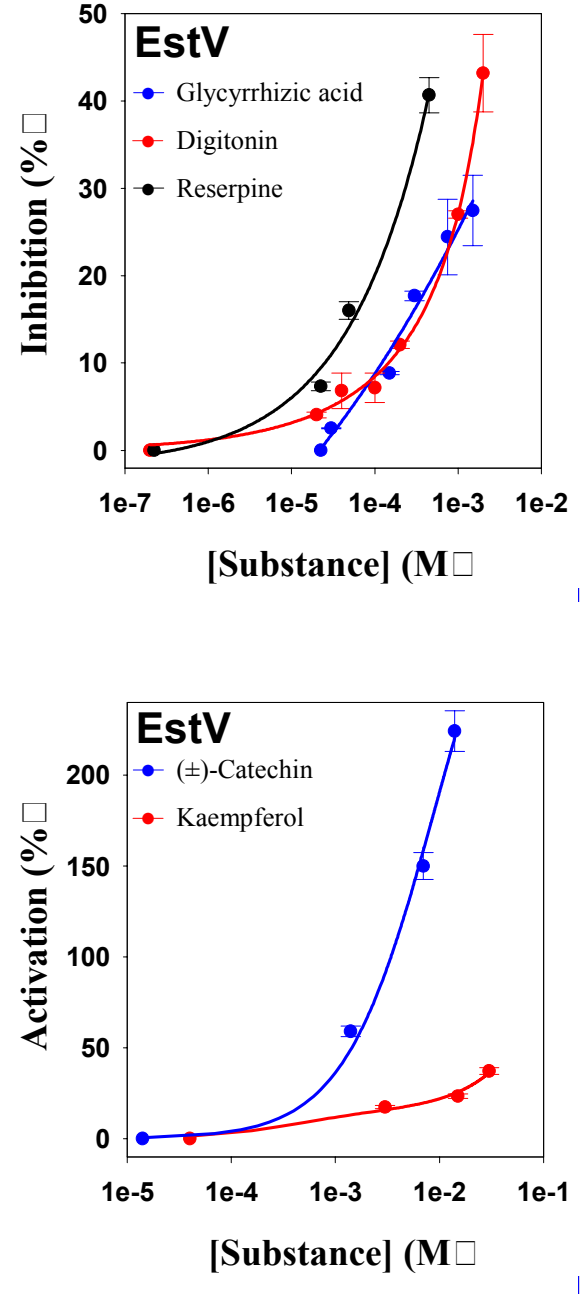

Figure 3: Ruiz et al.

FIGURE LEGENDS

Figure 1: Amino acid sequence and secondary structure prediction of EstV. Conserved pentapeptide of lipases containing the catalytic serine in bold, putative catalytic aminoacids undrlined. Pred: secondary structure prediction (C: coil -flat line-; H: α helix -cylinders- ; E: β strand -arrows-). Conf: confidence of the secondary structure predicted (from 0 -the lowest confidence- to 9 -the highest confidence-).

Figure 2: Cloning and purification of EstV. A: Hydrolysis halo produced by recombinant clone *E. coli* DH5α–pUC-EstV on CeNAN agar plates supplemented with tributyrin. B: Coomassie stained (left) SDS-PAGE and zymogram (rigth) of purified EstV using MUF-butyrate as a substrate. 1: broad-range MW protein marker; 2: EstV purified fraction, indicated by an arrow.

Figure 3: Inhibition and activation of EstV by natural substances. Substances displaying no activity on *H. pylori* EstV are not shown.

Propionibacterium acnes GehA lipase: cloning, characterization and inhibition by natural substances

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Running Title: Characterization and inhibition of *Propionibacterium acnes* lipase

Keywords: Lipase, *Propionibacterium acnes*, inhibition, saponins, flavonoids, alkaloids

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In preparation

Characterization and inhibition of bacterial lipases involved in pathogenesis.

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Running Title: Bacterial lipases involved in pathogenesis.

Keywords: Lipase, esterase, *Helicobacter pylori*, *Propionibacterium acnes*, inhibition

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