GENERAL
MATERIALS AND METHODS

Figure M.1 AKTA FPLC (Fast Protein Liquid Chromatograph).

♦ **Note:** the general materials and methods used in this PhD Thesis are explained in this section, whereas particular modifications of these procedures, and specific methods, are explained in the corresponding chapters.

1 REAGENTS

1.1 COMERCIAL SOURCES OF COMMONEST REAGENTS

1-9

3-HF (3-hydroxyflavone): Sigma

5-HF (5-hydroxyflavone): Sigma

A

Acetic acid (glacial): Panreac

Acetone: Sigma

Acetonitrile: Lab-Scan

Acrylamide: Bio-Rad

Agar: Scharlau Microbiology

Agarose (Low EEO): Roche

$AgNO_3$: Merck

Alkaline phosphatase: Roche

Ammonium sulphate: Merck

Ampicillin: Sigma

Anti-digoxigenin antibody linked to alkaline phosphatase: Roche

APS (ammonium persulphate): Merck

Arabic gum: Sigma

Aspidospermine: Simes Spa

B

$BaCl_2$: Merck

Bactotryptone: ADSA Micro

Bisacrylamide: Bio-Rad

Blocking reagent: Roche

Boric acid: Sigma

Bovine serum albumin: Sigma

Brilliant Blue Coomassie® R250: Sigma

Bromophenol blue: Bio-Rad

C

$CaCl_2$: Panreac

Candida rugosa lipase (CRL): Sigma (catalogue number L-1754)

Capric acid: Sigma

Carboxymethyl cellulose: Sigma

(±)-Catechin: Sigma

CeNAN tributyrin base agar: ADSA Micro

Chloroform: Merck

Citric acid: Merck

Cloned *Pfu* polymerase (and buffer): Stratagene

$CoCl_2 \cdot 6H_2O$: Merck

$CuSO_4$: Merck

D-E

D(+)-Glucose: Panreac

DIG DNA 10× labelling mixture: Roche

Digitonin: ICN Biomedicals Inc.

Dimethyl sulfoxide: Sigma

Dimethylformamide: Prolabo

dNTPs: Promega

EDTA (Titriplex®III): Merck

EGME: Merck

Ethanol: Merck

Ethidium bromide: Sigma

Ethyl acetate: Aldrich

F-G

Fe Cl_2: Panreac

$FeSO_4 \cdot 7H_2O$: Merck

GA (Glycyrrhizic acid): Sigma

Glutaraldehyde: Sigma

Glycerol: Panreac

Glycine: Bio-Rad

Gold-palladium: Aldrich

H-I-J

HCl: Panreac

$HgCl_2$: Prolabo

Imidazole: Merck

IPTG: Roche

Isoamylalcohol: Probus

Isopropanol: Prolabo

K-L

$K_2HPO_4 \cdot 3H_2O$: Merck

Kaempferol: Fluka

Kanamycin monosulphate (Kan): Sigma

KCl: Panreac

KH_2PO_4: Merck

Lactophenol blue: Bio-Rad

Lauric acid: Sigma

Lead citrate: Sigma

LiCl: Merck

Ligase (T4 DNA ligase): Bio-Labs

Liquid carbon dioxide: Sigma

Lysozyme: Merck

M

Meat peptone: ADSA micro

Methanol: Lab-Scan

$MgCl_2$: Merck

$MgSO_4 \cdot 7H_2O$: Merck

$MnCl_2$: Merck

$MnSO_4 \cdot H_2O$: Probus

MUF: Fluka

MUF-butyrate: Fluka

MUF-oleate: Fluka

Myristic acid: Sigma

N

Na_2CO_3: Panreac

Na_2HPO_4: Merck

NaCl: Panreac

$NaH_2PO_4 \cdot H2O$: Merck

NAI: Sigma

NaOH: Panreac

NBS: Sigma

NBT/BCIP stock solution: Roche

NH_4Cl: Merck

$NiCl_2$: Panreac

Nitrilotriacetic acid salt: Merck

NTA-Ni: □iagen

Nutrient broth: ADSA Micro

O-P-Q

Oat spelt xylan: Sigma

Olive oil (1º extra virgin): Carbonell

OsO_4: Sigma

Papaverine: Sigma

$Pb(CH_3COO)_2$: Merck

PGA (polygalacturonic acid): Sigma

Phenol: Fluka

PHMB: Sigma

Physostigmine: Sigma

Phytic acid: Sigma

Pilocarpine: Sigma

PMSF: Sigma

p-nitrophenol: Fluka

p-NP-derivatives: Fluka

Proteinase K: Merck

□S (*Quillaja* saponin): Sigma

R

Raubasine: Simes Spa

Reinforced Clostridial Agar (RCM): Scharlau Microbiology

Rescinnamine: Simes Spa

Reserpine: Sigma

Restriction nucleases and their reaction buffers: Roche/Bio-Labs

Rhodamine B: Sigma

Ringer ¼: ADSA Micro

RNAse A: Roche

S

Sabouraud-chloramphenicol agar: ADSA Micro

SDS: Merck

Sodium acetate: Merck

Sodium azide: Merck

Sodium citrate: Merck

Sodium laurylsarcosinate: Sigma

Sodium taurocholate: Sigma

Spurr resin: Sigma

Streptomycin sulphate: Sigma

Succinic acid: Merck

T

Taq Polymerase (and buffer): Biotools

TEMED: Bio-Rad

Tetracycline: Sigma

Thioglycolic acid: Merck

Tributyrin: Scharlau Microbiology

Trichloroacetic acid: Merck

Trifluoroacetic acid: Aldrich

Trigonelline: Sigma

Triolein: Scharlau Microbiology

Tris: Bio-Rad

Triton X-100: Sigma

Tryptone: ADSA micro

U-V-W

Uranyl acetate: Sigma

Urea: Merck

Water for HPLC: Lab-Scan

X-Y-Z

X-gal: Roche

Xylene cyanol: Bio-Rad

Yeast extract: Scharlau Microbiology

$ZnCl_2$: Merck

$ZnSO_4 \cdot 7H_2O$: Merck

β

β-Aescin: Sigma

β-Mercaptoethanol: Sigma

β-Naphtol: Fluka

β-Naphtyl laurate: Fluka

1.2 PREPARATION OF SPECIFIC SUBSTANCES

1.2.1 Preparation of antibiotics

Culture media were supplemented with concentrated solutions of the following antibiotics in order to select and maintain plasmid-containing *Escherichia coli* strains (Sambrook *et al*., 1989). Antibiotic solutions were sterilized by filtration (see General Materials and methods 2.1), and stored at −20 ºC.

Ampicillin (Ap□ prepared at a concentration of 100 mg ml^{-1} in bidistilled water plus the amount of NaOH necessary to achieve its complete dissolution. The final concentration used for pBR322 or pUC19 (and their derivatives) plasmid-containing *E. coli* strains was 50 μg ml^{-1}.

Kanamycin (Kan□ kanamycin monosulphate was prepared at a concentration of 25 mg ml^{-1} in bidistilled water. The final concentration used for *E. coli* strains containing pET28a (and its derivatives) plasmid was 125 μg ml^{-1} for BL21(DE3) strains, and 50 μg ml^{-1} for DH5α strains.

Tetracycline (Tc□ prepared at a concentration of 12.5 mg ml^{-1} in ethanol and stored light-protected. The final concentration used for *E. coli* XL1-Blue and pBR322-containg strains was 15 μg ml^{-1}.

1.2.2 Preparation of IPTG, X-gal, Rhodamine B and MUF-derivatives

Luria-Bertani agar was supplemented with IPTG (isopropyl-β-D-thiogalactopyranoside) and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) when the α-complementation system was used to differentiate *E. coli* XL1-Blue or *E. coli* DH5α strains containing recombinant pUC19 plasmids from those containing non-recombinant plasmids (Sambrook *et al*., 1989).

IPTG: prepared at a concentration of 20 mg ml^{-1} in bidistilled water, sterilized by filtration, and stored at −20 ºC. The final concentration used was 100 μg ml^{-1} for transformation assays, and 1 mM for induction of protein expression assays.

X-gal: prepared at a concentration of 200 mg ml^{-1} in dimethylformamide, and stored at −20 ºC. The final concentration used in transformation assays was 40 μg ml^{-1}.

Rhodamine B was added to culture media supplemented with lipid substrates to detect lipolytic activity by UV illumination (Kouker & Jaeger, 1987).

Rhodamine B: prepared at a concentration of 1 mg ml^{-1} (0.1% by w/v) in bidistilled water, sterilized by filtration, and stored at −20 ºC. The final concentration was 2 μg ml^{-1} ($2 \cdot 10^{-4}$% by w/v).

Detection and determination of lipolytic activity by using fluorogenic substrates (zymograms, fluorimetric assays, paper assays, etc) was performed using derivatives of 4-methylumbelliferone (MUF) (Díaz *et al*., 1999; Prim *et al*., 2003):

MUF: prepared at several concentrations in ethyleneglycol monomethylether (EGME), and stored at −20 ºC. It was used to prepare the calibration standards.

MUF-butyrate stock solution: prepared at a concentration of 25 mM in EGME, and stored at −20 ºC. The final concentration used was 100 μM.

MUF-oleate stock solution: prepared at a concentration of 100 mM in EGME, and stored at −20 ºC. The final concentration used was 400 μM.

2 MICROBIOLOGICAL METHODS

2.1 STERILIZATION OF MATERIALS AND SOLUTIONS

Glass and plastic material, culture media, etc, were sterilized by wet heat and pressure by autoclaving them at 1 atmosphere pressure for 20 min (10 min for highly concentrated sugar solutions in order to avoid caramelization) at 121 ºC.

Thermolabile solutions were "sterilized" by filtration through Millex® GS (Millipore) sterile nitrocellulose filters with a pore-diameter of 0.22 μm.

2.2 STRAINS, CULTURE MEDIA AND GROWTH CONDITIONS

The microorganisms used in this work are displayed in Table M.1, and the composition of the culture media used for their isolation, growing, or maintenance is detailed in Table M.2.

In general, liquid cultures were inoculated from plate cultures or previous liquid cultures (using 1:50 or 1:100 dilutions in fresh media) and were incubated under agitation at 200 r.p.m in a Certomat®R agitator (B. Braun). Microbial growth was determined by measuring culture absorbance at λ = 600 nm ($A_{600\ nm}$) in a Spectrophotometer Du® (Beckman Coulter).

Table M.1 Microorganisms and growth conditions used in this study.

A. STRAINS WITH BIOTECHNOLOGICAL POTENTIAL

Bacillus megaterium CECT 370 (ATCC9885□

- **Origin/Reference:** strain from the Spanish Type Culture Collection (CECT).
- **Features:** lipolytic strain.
- **Maintenance:** monthly cultures on Nutrient agar plates incubated O/N at 30 ºC.
- **Utilization:** analysis of its lipolytic system and cloning of *B. megaterium lipA* gene.

Bacillus sp. BP-6 (CECT 5670□

- **Origin/Reference:** isolated by Dr. A. Blanco and Dr. F.I.J. Pastor from rice field soil of Ebro's Delta River (Spain) by heat shock and subsequent enrichment culture on rice straw.
- **Features:** polysaccharide- and lipid-degrading strain.
- **Maintenance:** monthly cultures on Nutrient agar plates incubated O/N at 30 ºC.
- **Utilization:** analysis of its lipolytic system and cloning of *Bacillus* sp. BP-6 *lipA* gene.

Bacillus sp. BP-7 (CECT 5337□

- **Origin/Reference:** isolated from rice field soil of Ebro's Delta River (Spain) by heat shock and subsequent enrichment culture on rice straw (López *et al*., 1998).
- **Features:** lipase, cellulase, xylanase and pectinase activities. A cell-bound carboxylesterase has been cloned from this strain (Prim *et al*., 2001).
- **Maintenance:** monthly cultures on Nutrient agar plates incubated O/N at 30 ºC.
- **Utilization:** cloning of *Bacillus* sp. BP-7 *lipA* gene, and positive control in lipase assays.

Bacillus sp. CR-179; *Rhodococcus* sp. CR-53; and other CR- strains

- **Origin:** all these strains were isolated from three soil samples from a subtropical forest soil of Puerto Iguazú (Argentina); this study (see General Materials and Methods 2.3 and Chapter 2).
- **Features:** lipolytic and/or polysaccharide-degrading strains (see Chapter 2).
- **Maintenance:** in general, monthly cultures on their corresponding culture media and temperatures of isolation (see Chapter 2). *Bacillus* sp. CR-179: isolated at 42 ºC on Horikoshi-I agar and maintained on Luria-Bertani agar plates incubated O/N at 42 ºC; *Rhodococcus* sp. CR-53: isolated and maintained on Luria-Bertani agar plates incubated for 4−5 days at 20 ºC.
- **Utilization:** isolation, identification of their hydrolytic activities, determination of the lipolytic activities of the most active ones, and physiological and biochemical characterization of strains *Bacillus* sp. CR-179 and *Rhodococcus* sp. CR-53.

Paenibacillus sp. BP-23 (formerly *Bacillus* sp. BP-23□

- **Origin/Reference:** isolated from rice field soil of Ebro's Delta River (Spain) by heat shock and subsequent enrichment culture on rice straw (Blanco & Pastor, 1993; Sánchez *et al*., 2005).
- **Features:** lipase, cellulase, xylanase and pectinase activities. A cell-bound carboxylesterase has been cloned from this strain (Prim *et al*., 2000).
- **Maintenance:** monthly cultures on Nutrient agar plates incubated O/N at 30 ºC.
- **Utilization:** positive control in cellulase, xylanase and pectinase activity plate assays.

B. PATHOGENIC STRAINS

Helicobacter pylori 26695

- **Origin/Reference:** this strain (Tomb *et al*., 1997) was kindly provided by N. □ueralt and Dr. R. Araujo.
- **Features:** virulent wild-type strain whose genome was sequenced by Tomb *et al*. (1997).
- **Maintenance:** this strain was not stored or maintained. Fresh cultures (grown on Columbia agar supplemented with 5% lysed defibrinated horse blood, and incubated for 4 days at 37 ºC under a microaerobic atmosphere (N_2:CO_2:O_2; 75:15:10, by volume)) were provided each time by N. □ueralt.
- **Utilization:** cloning of EstV carboxylesterase-coding gene (*HP0739*).

Propionibacterium acnes P-37

- **Origin/Reference:** this strain (Gribbon *et al*., 1993) was kindly provided by Dr. M.D. Farrar and Dr. K.T. Holland.
- **Features:** strain from which *P. acnes* lipase gene (*gehA*) was isolated by Miskin *et al*. (1997).
- **Maintenance:** monthly cultures on Reinforced Clostridial Agar plates incubated for 24 h at 34−37 ºC in an anaerobic jar with an atmosphere of N_2:CO_2:H_2 (80:10:10, by volume) achieved by using the Anaero*Gen*™ system (Oxoid), and confirmed with an Anaerobic indicator BR35 (Oxoid). Long-term storage at −80 ºC was performed on PBS + 40% glycerol (v/v).
- **Utilization:** cloning of *P. acnes* lipase (*gehA*) gene.

C. GENETICALLY MODIFIED STRAINS

Bacillus subtilis BCL 1050

- **Reference and features:** Dartois *et al*. (1994): *est*::Cm *ΔlipA nprR2 nprE18 aprA3*.
- **Maintenance:** monthly cultures on Nutrient agar plates incubated O/N at 30 ºC.
- **Utilization:** negative control in lipolytic activity assays of *Bacillus* strains.

Bacillus subtilis MB216

- **Reference and features:** Lampen *et al*. (1986): *Stp leuA8 arg-15 thrA recE4 hsrR hsrM*.
- **Maintenance:** monthly cultures on Nutrient agar plates incubated O/N at 30 ºC.
- **Utilization:** positive control in assays for PCR amplification of *Bacillus* lipase-coding genes.

Escherichia coli strains

- **Reference and genotype:**
  - *E. coli* 5K (Godessart *et al*., 1988): F^- r_k^- m_k^- *rpsL thr thi leu lacZ*.
  - *E. coli* BL21(DE3) (Studier & Moffatt, 1986): *hsd*S *gal* (*λc*I*ts*857 *ind*1 *S*am7 *nin*5 *lac*UV5-T7 gene*1*.
  - *E. coli* DH5α (Hanahan, 1983): *supE44 ΔlacU169* (*ϕ80dlacZΔM15*) *hsdR17*(r_k^- m_k^+) *recA1 endA1 gyrA96 thi-1 relA1*.
  - *E. coli* XL1-Blue (Bullock *et al*., 1987): *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′*proAB lacI*^{q}Z*ΔM15* Tn*10* (Tet^r)].
- **Maintenance:** monthly cultures on Luria-Bertani agar plates incubated O/N at 37 ºC.
- **Utilization:** host strains for cloning and expression of lipase-coding genes.

Escherichia coli recombinant clones

- **Reference and features:**
  - *E. coli* 5K–pBR-BP6LipA (contains *Bacillus* sp. BP-6 *lipA*): this study.
  - *E. coli* 5K–pBR-BP7LipA (contains *Bacillus* sp. BP-7 *lipA*): this study.
  - *E. coli* 5K–pBR-BMLipA (contains *B. megaterium* CECT370 *lipA*): this study.
  - *E. coli* 5K–pBR-BSLipA (contains *B. subtilis* MB216 *lipA*): Sánchez *et al*., 2002.
  - *E. coli* 5K–pL37 (contains *Paenibacillus* sp. BP-23 *estA*): Prim *et al*., 2000.
  - *E. coli* BL21(DE3) –pET28-EstV (contains *H. pylori estV* (*HP0739*)): this study.
  - *E. coli* DH5α–pET28-EstV (contains *H. pylori estV* (*HP0739*)): this study.
  - *E. coli* DH5α–pUC-EstV (contains *H. pylori estV* (*HP0739*)): this study.
  - *E. coli* XL1-Blue–pUC-E1 (contains *Bacillus* sp. BP-7 *estA1*): Prim *et al*., 2001.
  - *E. coli* XL1-Blue–pUC-GehA (contains *P. acnes gehA*): this study.
- **Maintenance:** monthly cultures on Luria-Bertani agar plates supplemented with Ap (Kan for clones containing pET28a plasmid-derivatives), and incubated O/N at 37 ºC.
- **Utilization:** expression of lipolytic genes.

♦ **Note.** Unless otherwise stated:

- strains were grown under aerobic conditions.
- microbial plate cultures were stored at 4 ºC for one month.
- long-term storage of microorganisms was performed by adding 20% sterile glycerol (v/v) to the corresponding liquid cultures and subsequent freezing at −80 ºC.

Table M.2 Culture media and saline solutions.

CeNAN tributyrin base agar (ADSA Micro)

- **Properties:** rich medium especially suitable for plate assays of lipolytic activity detection.
- **Composition:** 5.5 g peptone, 3.5 g yeast extract, 15 g agar, per L; pH 7.5.
- **Others:** it was supplemented with a lipid substrate (tributyrin, triolein, or olive oil) at a concentration of 1% (v/v). Tributyrin or triolein were autoclaved together with the medium, whereas olive oil was autoclaved separately. Addition of $2 \cdot 10^{-4}$% (w/v) "sterile" Rhodamine B and strong agitation were performed before pouring the medium into the Petri dishes.

Horikoshi-I agar (Horikoshi, 1999)

- **Properties:** rich medium especially suitable for the isolation of alkalophilic microorganisms.
- **Composition:** 10 g D(+)-glucose, 5 g bactotryptone, 5 g yeast extract, 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.
- **Others:** D(+)-glucose was sterilized separately by autoclaving it for 10 min.

Luria-Bertani broth (LB) (Sambrook *et al.*, 1989)

- **Properties:** rich medium suitable for most chemoheterotrophic microorganisms.
- **Composition:** 10 g bactotryptone, 5 g yeast extract, 10 g NaCl, per L; pH 7. Plus 15 g agar per L for **LB agar**.
- **Others:** after autoclaving, it was supplemented with Ap, Kan, Tc, IPTG and/or X-gal, when necessary. It was supplemented with 0.4% (w/v) oat spelt xylan (autoclaved separately) for xylanase plate assays, and with 0.5% (w/v) carboxymethyl cellulose (autoclaved together with the medium) for cellulase plate assays.

Mineral basal medium agar (Hareland *et al.*, 1975)

- **Properties:** synthetic medium with neither carbon nor energy source.
- **Composition:** 500 ml Solution A + 500 ml Solution B plus 15 g agarose per L; pH 7.2. Both solutions are prepared separately using bidistilled water.
  - Solution A composition: 4.25 g of $K_2HPO_4 \cdot 3H_2O$, 1 g $NaH_2PO_4 \cdot H2O$, 2 g NH_4Cl, per L.
  - Solution B composition: 123 mg Nitrilotriacetic acid salt, 0.2 g $MgSO_4 \cdot 7H_20$, 120 mg $FeSO_4 \cdot 7H2O$, 3 mg $MnSO_4 \cdot H2O$, 3 mg $ZnSO_4 \cdot 7H2O$, 1 mg $CoCl_2 \cdot 6H_2O$, per L.
- **Others:** it was supplemented with 1% v/v tributyrin, triolein, or olive oil for assays of bacterial growth using a single carbon and energy source.

Nutrient broth (Scharlau Microbiology)

- **Properties:** rich medium suitable for *Bacillus* and most chemoheterotrophic microorganisms.
- **Composition:** 5 g peptone, 2 g yeast extract, 5 g NaCl, 1 g meat extract, per L; pH 7.4. Plus 15 g agar per L in **Nutrient agar**.
- **Others:** it was supplemented with 1% (w/v) polygalacturonic acid (PGA; autoclaved together with the medium) for pectinase plate assays.

Phosphate Buffered Saline (PBS) (Sambrook *et al*., 1989)

- **Properties:** saline solution for eukaryotic and prokaryotic cells.
- **Composition:** 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4; pH 7.4.
- **Others:** it was supplemented with 40% (v/v) glycerol for *P. acnes* storage at −80 ºC.

Reinforced Clostridial Agar (RCM; Scharlau Microbiology)

- **Properties:** rich medium suitable for the cultivation of anaerobic microorganisms. It is free from inhibitors and contains cysteine as reducing agent.
- **Composition:** 10 g meat extract, 10 g peptone from casein, 3 g yeast extract, 5 g D(+)-glucose, 1 g starch, 5 g NaCl, 3 g sodium acetate, 0.5 g L-cysteinium chloride, 12.5 g agar, per L; pH 6.8.

Ringer ¼ (ADSA Micro)

- **Properties:** isotonic solution for maintaining the viability of microorganisms without allowing their replication.
- **Composition:** 2.25 g NaCl, 105 mg KCl, 0.12 g $CaCl_2$, per L; pH 7.2.

Sabouraud-agar (ADSA Micro)

- **Properties:** rich medium suitable for fungi maintenance due to its high glucose concentration and low pH.
- **Composition:** 5 g tryptone, 5 g meat peptone, 40 g D(+)-glucose, 15 g agar, per L; pH 5.6. **Sabouraud-broth** was prepared following this composition but without agar addition.

Sabouraud-chloramphenicol agar (ADSA Micro)

- **Properties:** rich medium suitable for the isolation of fungi from bacteria containing samples due to its high glucose concentration and low pH, as well as due to the presence of chloramphenicol (mainly toxic for prokaryotic ribosomes).
- **Composition:** 5 g tryptone, 5 g meat peptone, 40 g D(+)-glucose, 0.5 g chloramphenicol, 15 g agar, per L; pH 5.6.

♦ **Notes:** • Unless otherwise stated, media were prepared in distilled water and sterilized by autoclaving.
• pH was adjusted with NaOH or HCl solutions when necessary.

2.3 ISOLATION OF MICROORGANISMS FROM SOIL SAMPLES

Microorganisms from soil samples collected from a subtropical forest in Iguazú rainfalls (Puerto Iguazú, Argentina) were isolated essentially as described by Madrid *et al*. (1997). Microorganisms were extracted by suspending 1 g of each soil sample in 20 ml saline solution Ringer ¼. 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 ºC to isolate only spore-forming bacteria, whereas the other sample remained untreated in order to isolate spore-forming and nonspore-forming microorganisms.

These samples were used for isolation of aerobic and facultative anaerobic microorganisms by means of serial dilutions in Ringer ¼. 0.1 ml of each dilution were spread on agar plates containing five different culture media, and the resulting plates were incubated in duplicate for 1−7 days at three different temperatures (20 ºC, 30 ºC and 42 ºC). According to the microorganisms pursued, the five culture media used were: LB agar, Nutrient agar, Sabouraud-chloramphenicol agar, Horikoshi-I agar and CeNAN-olive oil agar.

For each medium and temperature of isolation, those colonies displaying different morphological properties were selected and their Gram stain performed. A pure culture of each strain was obtained and maintained using the same culture conditions of isolation (temperature and culture medium), with the exception of the isolates obtained in Sabouraud-chloramphenicol agar and Horikoshi-I agar, which were maintained in Sabouraud agar and LB agar, respectively.

2.4 IDENTIFICATION OF BACTERIAL STRAINS

Preliminary identification of the strains *Bacillus* sp. CR-179 and *Rhodococcus* sp. CR-53 was performed through morphological, physiological and molecular assays.

Motility test, lactophenol blue stain for fungal strains, Gram, acid fast, spore, capsule and other specific bacterial stains were performed on each strain, and cells were observed at × 1000 under a B202 double beam optical microscope (Olympus).

Transmission (TEM) and scanning (SEM) electron microscopy were performed at the Serveis Científico Tècnics of the Universitat de Barcelona using cells from strain CR-179 grown in LB for 48 h at 42 ºC, and cells from strain CR-53 grown in LB for 4 days at 20 ºC. The samples were fixed by mixing 1 volume sample with 1 volume of fixation solution (0.1 M phosphate buffer (pH 7.4) containing 5% (v/v) glutaraldehyde), followed by incubation for 1 h at 4 ºC. The samples were then centrifuged at 10000 ×g for 5 min at 4 ºC and the supernatants removed. After that, the samples were covered with fixation solution and maintained at 4 ºC. Fixed samples were then 3-times washed for 10 min in 0.1 M phosphate buffer (pH 7.4), post-fixed in 1% OsO_4 for 1 hour at 4 ºC, 3-times washed for 10 min in bidistilled water, and harvested (centrifugation at 10000 ×g for 5 min at 4 ºC). The supernatants were then removed, and the samples were prepared for being dehydrated.

TEM samples were dehydrated in the following acetone series (percentages in v/v): 1 × 50% (10 min), 2 × 70% (10 min), 3 × 90% (10 min), 3 × 96% (10 min) and 3 × 100% (15 min). The samples were then infiltrated in an increased series of Spurr resin in acetone, transferred to 100% resin, and polymerized at 55 ºC for 24 h. Thin sections were cut on a Ultracut E ultramicrotome (Reichert-Jung) and collected on copper grids. The resulting sections were stained first with 2% (w/v) uranyl acetate for 10–12 min, and then with 1% lead citrate for 3–5 min, and were visualized on a JEM 1010 transmission electron microscope (JEOL).

Post-fixed SEM samples were dehydrated in the following ethanol series (percentages in v/v): 1 × 50% (10 min), 2 × 70% (10 min), 3 × 90% (10 min), 3 × 96% (10 min) and 3 × 100% (15 min). The ethanol was then replaced by liquid carbon dioxide under pressure using a CPD 7501 critical point drier (POLARON). Liquid carbon dioxide was then evaporated raising the temperature. The dried samples were mounted on stubs and sputter-coated with a heavy metal (gold-palladium) to convey electrical conductivity. Finally, the samples were visualized in a DSM 940 scanning electron microscope (ZEISS).

Determination of the physiological properties of strains CR-179 and CR-53 was performed according to the microbiological tests recommended for the genus *Bacillus* and other Gram-positive bacteria (Gordon *et al.*, 1973) and, in the case of strain CR-53, for actinomycetes (Goodfellow *et al*., 1998). These tests included, among other assays, aerobic or anaerobic growth, pH range of growth, methyl-red and Voges–Proskauer tests, oxidase, catalase, hydrolysis of casein, gas and acid production from lactose, use of citrate, nitrate reduction, indole production and hydrolysis of polysaccharides. API 20E and API 50CH galleries (BioMèrieux) were also used.

Molecular analysis of these strains was performed by amplification and sequencing of their 16S rDNA, followed by sequence analysis and computing of matrices of evolutionary distances (General Materials and Methods 3.5, 3.6 and 3.7, respectively).

3 DNA METHODS

3.1 PLASMIDS USED

Plasmids used in this study are shown in Table M.3.

Table M.3 Plasmids used in the present work.

PLASMID	RELEVANT GENOTYPE/PHENOTYPE	REFERENCE
pBR322	Cloning vector: Ap^r Tc^r	Bolivar *et al*., 1977
pET28a	Expression vector (His-tag system containing a thrombin cleavage site): Kan^r	Studier & Moffatt, 1986; Novagen
pUC19	Cloning vector: Ap^r, *lacZ lacI*	Yanisch-Perron *et al*., 1985
pBR-BP6LipA	Ap^r Tc^s Est^+: *Bacillus* sp. BP-6 *lipA* gene cloned into *Eco*RV site of pBR322	This study
pBR-BP7LipA	Ap^r Tc^s Est^+: *Bacillus* sp. BP-7 *lipA* gene cloned into *Eco*RV site of pBR322	This study
pBR-BMLipA	Ap^r Tc^s Lip^+: *B. megaterium* CECT370 *lipA* gene cloned into *Eco*RV site of pBR322	This study
pBR-BSLipA	Ap^r Tc^s Lip^+: *Bacillus subtilis* MB216 *lipA* gene cloned into *Eco*RV site of pBR322	Sánchez *et al*., 2002
pL37	Ap^r Tc^s Est^+: *Sau*3AI–*Sau*3AI genomic DNA fragment of *Bacillus* sp. BP-7, containing *estA* gene, cloned into *Bam*HI site of pBR322	Prim *et al*., 2000
pET28a-EstV	Kan^r Est^+: *H. pylori estV* (*HP0739*) cloned into *Nde*I–*Pst*I site of pET28a	This study
pUC-E1	Ap^r Est^+: *Sau*3AI–*Sau*3AI *Bacillus* sp BP-7 genomic DNA fragment containing *estA1* gene cloned into *Bam*HI site of pUC19	Prim *et al*., 2001
pUC-EstV	Ap^r Est^+: *H. pylori estV* (*HP0739*) cloned into *Sma*I site of pUC19	This study
pUC-GehA	Ap^r Geh^+: *P. acnes gehA* cloned into *Xba*I–*Pst*I site of pUC19	This study

3.2 CLEANING AND CONCENTRATION OF DNA

- **Phenol:chloroform treatment** (Sambrook *et al*., 1989) was generally performed to remove proteins or other contaminants from DNA solutions. DNA was mixed with 1 volume of phenol (equilibrated at pH 8 and containing 1% (w/v) 8-hydroxyquinoline). After agitation, sample was centrifuged at 13000 ×g for 2 min at room temperature (RT), and the aqueous phase was recovered. This process was repeated using phenol:chloroform:isoamylalcohol (25:24:1, by volume) first, and chloroform:isoamylalcohol (24:1, by volume) afterwards.

- **Ethanol precipitation** (Sambrook *et al*., 1989) was used to concentrate and clean DNA solutions. DNA was mixed with 2 volumes of absolute ethanol at −20 ºC and 0.1 volumes of 3 M sodium acetate (pH 4.8), incubated for 30 min at −20 ºC, and centrifuged at 13000 ×g for 20 min at 4 ºC. The supernatant was discarded and the pellet was washed twice by addition of 250 µl of 70% (v/v) ethanol at −20 ºC, followed by centrifugation at 13000 ×g for 10 min at 4 ºC. The supernatant was discarded and the pellet was dried using a speed-vacuum centrifuge (Eppendorf). Dried DNA was suspended in an appropriate volume of distilled water.

- **The commercial kit Wizard® PCR Preps (Promega** was used in certain occasions to carry out the direct purification of DNA from PCR reactions yielding a single amplification product. This kit is based on DNA binding to an ionic exchange resin, followed by resin binding to a column, column washing, and subsequent DNA elution in reduced ionic strength conditions.

3.3 ISOLATION OF DNA

Two different methods were used for isolating plasmid DNA:

3.3.1 Minipreparation of plasmid DNA

Isolation of plasmid DNA from *E. coli* recombinant clones was performed by the LiCl method (Martínez & de la Cruz, 1988). The composition of the solutions used is detailed in Table M.4.

Overnight cultures (5 ml) of the corresponding clones were centrifuged at 5000 ×g for 10 min at 4 ºC to recover the cells. The pellets were suspended in 200 µl of solution I at 4 ºC, and incubated for 5 min in ice before adding 400 µl of solution II. The samples were then mixed by gentle inversion and subsequently incubated for 5 min at 4 ºC. After that, 300 µl of solution III was added to the samples, which were mixed and subsequently incubated for 5 min at 4 ºC. The samples were then centrifuged at 13000 ×g for 15 min at 4 ºC, their supernatants were recovered, and 500 µl of isopropanol were added to each sample before incubating them for 5 min at RT. Then, they were centrifuged again at 13000 ×g for 5 min at 4 ºC for DNA precipitation. The supernatants were discarded, and the DNA pellets were dried in a speed-vacuum centrifuge before being suspended in 200 µl of TE and 200 µl of LiCl 5 M. After 5-min incubation at –20 ºC, the samples were centrifuged at 13000 ×g for 5 min at 4 ºC. Finally, the supernatants were recovered and ethanol precipitated.

Table M.4 Solutions used for the minipreparations of plasmid DNA.

Solution I (suspension solution, 4 ºC) • 25 mM Tris–HCl (pH 8) • 50 mM D(+)-glucose • 10 mM EDTA (pH 8)	**Solution III** • 3 M sodium acetate (pH 4.8)
Solution II (lysis solution) • 0.2 M NaOH • 1% (w/v) SDS	**TE** • 10 mM Tris–HCl (pH 8) • 1 mM EDTA (pH 8)

3.3.2 Isolation of plasmid DNA using commercial columns

Isolation of large amounts of highly pure plasmid DNA was performed using the commercial kit □iagen-tip 100 MidiPrep (□iagen) according to the manufacturer's specifications. This system is based on an alkaline lysis followed by plasmid DNA binding to an anionic column, column washing, and subsequent DNA elution.

3.3.3 Isolation of genomic DNA

Genomic DNA was obtained essentially as described by Sambrook *et al*. (1989), and the composition of solutions used is detailed in Table M.5.

Table M.5 Solutions used for the isolation of genomic DNA.

TE2 • 50 mM Tris–HCl (pH 7.5) • 50 mM EDTA (pH 8) **TE3** • 50 mM Tris–HCl (pH 7.5) • 1 mM EDTA (pH 8)	**STEP solution** • 50 mM Tris–HCl (pH 7.5) • 0.2 M EDTA (pH 8) • 0.5 % (w/v) SDS • 1 mg ml^{-1} proteinase K

Overnight cultures (50 ml) of the corresponding strains were harvested by centrifugation at 5000 ×g for 10 minutes at 4 ºC. The pellets were suspended in 7.125 ml of TE2 solution before adding 375 µl of lysozyme (final concentration: 1 mg ml^{-1}). After 30-min incubation at 37−50 ºC, 1.5 ml of STEP solution were added to the samples, which were incubated at 4 ºC until clarification was observed (30−45 min). Then, the samples were treated with phenol:chloroform, and the DNA solutions obtained were treated by a short ethanol precipitation consisting in the addition of 2.5 volumes of absolute ethanol at −20 ºC and 0.1 volumes of 3 M sodium acetate (pH 4.8).

Precipitated DNAs were immediately recovered with a glass rod, transferred to 1 ml of solution TE3, and incubated O/N at 4 ºC. Next step consisted in the addition of 100 µg ml^{-1} (final concentration) RNAse A to the samples, which were incubated for 10−15 min at 30 ºC, and subsequently treated with phenol:chloroform. Finally, after a short ethanol precipitation, DNAs were again recovered with a glass rod, and dissolved in 500 µl of bidistilled water.

3.4 DNA GEL ELECTROPHORESIS

3.4.1 Electrophoresis

DNA was analyzed using agarose gels as is described by Sambrook *et al*. (1989). The composition of the solutions used is detailed in Table M.6.

DNA samples were prepared for electrophoresis by adding Loading buffer 6×. Then, samples were loaded into an agarose gel prepared, depending on the DNA molecular size, at a concentration of 0.8−2% (w/v, in TBE 1× or TAE 1× buffer). Finally, gel running was performed in a horizontal Mini-Sub®Cell GT tray (Bio-Rad) filled with TBE 1× or TAE 1× buffer, using a voltage of 75−120 volts generated by a PAC300 power supply (Bio-Rad).

Table M.6 Solutions used for DNA gel electrophoresis.

Loading buffer 6×	**TBE 1× (pH 8.3**	**TAE 1× (pH 7.6**
• 0.3% (w/v) bromophenol blue	• 90 mM Tris	• 40 mM Tris
• 0.3% (w/v) xylene cyanol	• 90 mM boric acid	• 40 mM acetic acid
• 72% (v/v) glycerol	• 2 mM EDTA (pH 8)	• 1 mM EDTA (pH 8)

3.4.2 DNA staining and visualization

DNA molecules separated in agarose gels were visualized by gel staining for 15−20 min in distilled water containing 75 µl ml^{-1} ethidium bromide, and subsequent irradiation with UV light (310 nm) using an ImageMaster® VDS (Pharmacia biotech). When DNA was required for further assays, visualization was performed using a UV-lamp (Black-Ray) of λ = 366 nm to reduce DNA damage.

3.4.3 DNA size estimation using molecular weight markers

DNA size was estimated using the SE□AID software by comparison of their migration length in agarose gels with that of the following molecular markers:

- **λ DNA/*Hind*III marker** (Promega): bacteriophage λ genome digested with *Hind*III. Range: 23−0.5 kb.
- **Φ DNA/*Hae*III marker** (Promega): bacteriophage ϕX174 genome digested with *Hae*III. Range: 1350−72 bp.
- **100 bp Ladder marker** (Biotools): bacteriophage pMLX genome digested with *Eco*147I and *Pvu*I. Range: 1031−80 bp.

3.4.4 Isolation of DNA fragments from agarose gels

Isolation of DNA from defined agarose gel bands was performed to purify DNA molecules suitable for cloning, sequencing, etc, when the DNA solutions contained several DNA molecules of different sizes. Two different methods were used:

3.4.4.1 Electroelution

DNA electroelution was performed as previously described (Sambrook *et al*., 1989) and used to purify high amounts of DNA.

DNA molecules were separated by electrophoresis in TAE 1× agarose gels. After gel staining and visualization, the part of gel containing the desired band was cut out with a scalpel and placed into a dialysis membrane (Sigma) filled with 1−2 ml of TAE 1×, which was then placed into an electrophoresis tray with TAE 1×. After 30−45-min electrophoresis at 75 volts, the polarity was exchanged and 2 pulses of 30 s were carried out. Finally, membrane TAE 1× containing the DNA was recovered, and the DNA was purified by phenol:chloroform treatment and ethanol precipitation.

3.4.4.2 Isolation of DNA fragments from agarose gels using commercial kits

Commercial kit □iaexII (□iagen) was used for fast purification of low amounts of DNA following the manufacturer's specifications. This method is based in melting the cut agarose fragments in a buffer containing silicagel particles. DNA binds to these particles and, after several washing steps, is eluted by lowering the ionic strength.

3.5 DNA AMPLIFICACION BY PCR

PCR (Polymerase Chain Reaction; Sambrook *et al*., 1989) was used for the *in vitro* synthesis of DNA molecules. Amplifications were usually performed by direct PCR, although inverse PCR (IPCR) amplifications were also performed.

3.5.1 Direct PCR

Direct PCR (Figure M.2) consisted in the amplification of the central region of DNA sequences located between two external primers whose extension proceeded towards the center of the molecule.

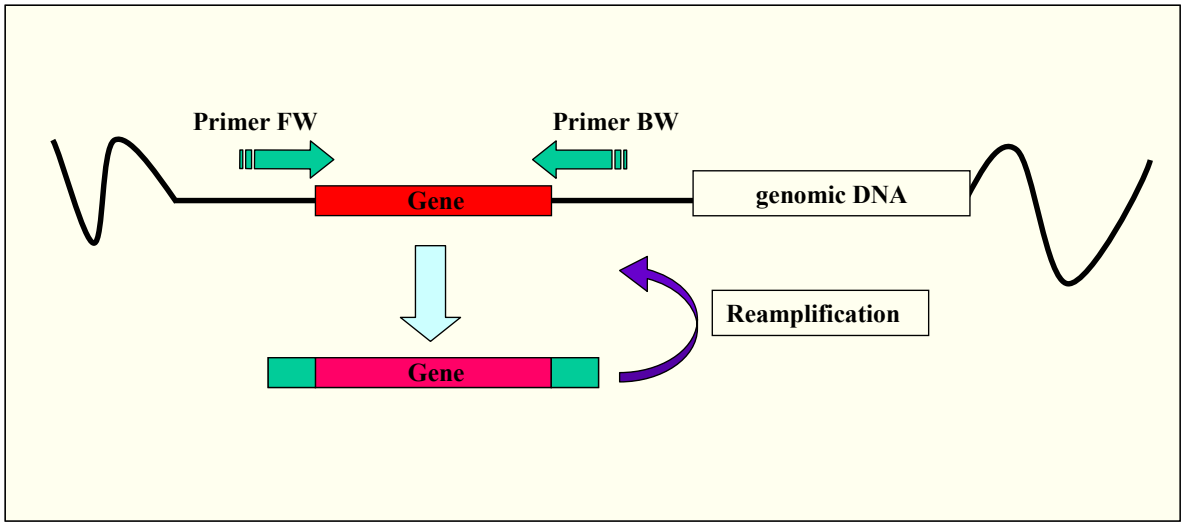

Figure M.2 Direct PCR amplification.

3.5.2 Inverse PCR

Inverse PCR (IPCR) was performed essentially as described (Ochman *et al*., 1988). Genomic DNA was first digested and re-ligated to obtain circular DNA molecules. Then, IPCR (Figure M.3) was performed using such circular molecules as templates. The process consisted in the amplification of the DNA sequences located 5' and 3' of two central primers whose extension proceeded towards the external ends of the desired DNA molecule. This system was used to determine the 5' and 3' sequences of *Bacillus*–lipase genes whose central region was known (see Chapter 1).

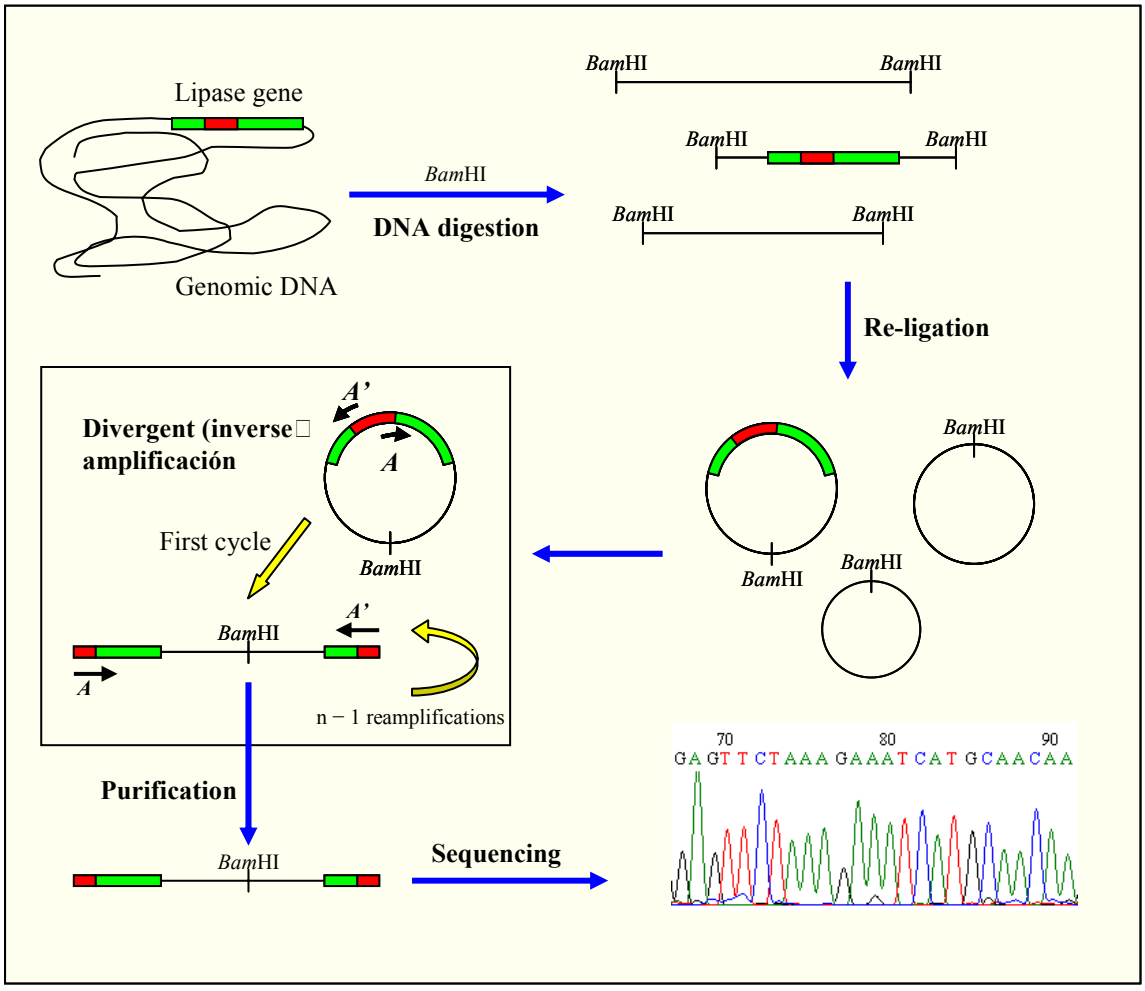

Figure M.3 General process of inverse PCR.

The scheme represents the general process of IPCR. Genomic DNA was digested (using *Bam*HI in the example) and re-ligated under conditions favouring intramolecular re-ligation. Then, divergent primers (A and A'), specific for the known central region (red) of the target DNA, were used for inverse amplification (IPCR) of the unknown 5' and 3' external regions (green) of the desired gene (one amplification per cycle). The resulting linear DNA molecule was then re-amplified in the subsequent cycles from the same A and A' primers by direct PCR. Finally, the resulting DNA was purified and sequenced to obtain the unknown nucleotide external sequence of the gene.

3.5.3 PCR conditions

Amplification reactions were performed in a GeneAMP PCR System 2400 thermocycler (Perkin Elmer). The primers used are detailed in Table M.7, whereas Table M.8 contains the reaction mixture components and the amplification programs used for the two types of thermostable polymerases used:

***Taq* polymerase:** highly-processive DNA polymerase without 3'→5' exonuclease proof-reading activity. This enzyme generates T-protuberant ends, and was used for routine amplifications.

Cloned *Pfu* polymerase: DNA polymerase used for high-fidelity amplifications due to having 3'→5' exonuclease proof-reading activity, but less processive than *Taq* polymerase. It generates blunt ends, thus it was used for blunt-end PCR cloning.

Table M.7 Primers used for amplification/sequencing reactions.

A. AMPLIFICATION OF *Bacillus*-related LIPASES

Consensus primers for *B. subtilis lipA* family (Chapter 1□

- **Primer FW:** FWSUB (5'-GAT ATT GTG GCT CAT AGT ATG GGC GG-3') Tm^1 = 64.4 ºC
- **Primer BW:** BKSUB (5'-GGC CTC CGC CGT TCA GCC CTT C-3') Tm^1 = 69 ºC
- **Reaction :** amplification of the central region (~300 bp) of *B. subtilis lipA*–related genes, Tm^2 = 42−55 ºC (usually 50 ºC)
- **Reference:** this study (designed by Dr. A. Blanco)

Consensus primers for *Gb. thermocatenulatus* lipase family (Chapter 1 and 2□

- **Primer FW:** FWTHER (5'-GTC GAT TAT GGC GCG GCA CAT GCG G-3') Tm^1 = 70.7 ºC
- **Primer BW:** BKTHER (5'-CCG TCA TGA GGT GTG GCA ATT GTT G-3') Tm^1 = 64.2 ºC
- **Reaction:** amplification of the central region (~300 bp) of *Gb. thermocatenulatus* lipase–related genes, Tm^2 = 42−55 ºC (usually 50 ºC)
- **Reference:** this study (designed by Dr. A. Blanco)

***B. megaterium* lipase inverse primers (Chapter 1□**

- **Primer FW:** FWMEGA (5'-GCT TTC AAA TAG TCA GGT TAA CGC G-3') Tm^1 = 51 ºC
- **Primer BW:** BKMEGA (5'-CCC TCC TAA CGT CAC GAC ATT TTC) Tm^1 = 52 ºC
- **Reaction:** inverse amplification of *B. megaterium lipA* gene, Tm^2 = 50 ºC.
- **Reference:** this study

***Bacillus* sp. BP-6 lipase inverse primers (Chapter 1□**

- **Primer FW:** FWBP6 (5'-AGC CAA GTC AAC GCC TAT ATC AAA G) Tm^1 = 51 ºC
- **Primer BW:** BKBP6 (5'-CCC ACC GAG CGT GAC AAC ATT TTG) Tm^1 = 54 ºC

- **Reaction:** inverse amplification of *Bacillus* sp. BP-6 *lipA* gene, Tm^2 = 50 ºC.
- **Reference:** this study

B. subtilis lipA primers (Chapter 1 and 2□

- **Primer FW:** FWBSLA (5'-TCT **AGA GGA GGA** TAT T*AT G*AA ATT TG-3'; *Xba*I site underlined, Shine-Dalgarno region in bold, start codon in italics) Tm^1 = 47 ºC
- **Primer BW:** BWBSLA (5'-GCA TGC CA**T TA**A TTC GTA TTC TGG CC-3'; *Sph*I site underlined, stop–complementary codon in bold) Tm^1 = 53 ºC
- **Reaction :** amplification of *B. subtilis lipA* gene, Tm^2 = 40−50 ºC
- **Reference:** Sanchez *et al*., 2002

B. subtilis lipB primers (Chapter 1 and 2□

- **Primer FW:** FWYfiP (5'-GTC TA**G AGG GGA** ATA AAC *GTG* AAA AAA G-3'; *Xba*I site underlined, Shine-Dalgarno region in bold, start codon in italics) Tm^1 = 52 ºC
- **Primer BW:** BKYfiP (5'-GGC ATG CAA GAT A**TT A**AT TTG TAT TGA GG-3'; *Sph*I site underlined, stop–complementary codon in bold) Tm^1 = 51 ºC
- **Reaction :** amplification of *B. subtilis lipB* (*YfiP*) gene, Tm^2 = 42−52 ºC
- **Reference:** this study

B. AMPLIFICATION OF LIPASES INVOLVED IN PATHOGENESIS

H. pylori estV–promoter primers (Chapter 5□

- **Primer FW:** HP0739FW (5'-TCC CTG CAG TTT GGC CAA TT-3'; *Xba*I site underlined) Tm^1 = 52 ºC
- **Primer BW:** HP0739BW (5'-CAG TCT AGA CAC CCC TAA AGG C-3'; *Pst*I site underlined) Tm^1 = 52 ºC
- **Reaction :** amplification of *H. pylori estV* (*HP0739*) gene including upstream (132 bp) and downstream (166 bp) regions, Tm^2 = 42−44 ºC
- **Reference:** this study

H. pylori estV–ORF primers (Chapter 5□

- **Primer FW:** 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) Tm^1 = 48 ºC
- **Primer BW:** HPESTBW (5'-CGC TAA ATG GAT CCA A**CT AA**G AC-3'; *Bam*HI site underlined, stop–complementary codon in bold) Tm^1 = 48 ºC
- **Reaction :** amplification of *H. pylori estV* (*HP0739*) gene, Tm^2 = 48 ºC
- **Reference:** this study

P. acnes gehA–promoter primers (Chapter 5□

- **Primer FW:** PALIPFW (5'-TTT CTG CAG GCT ACC CTT TTC G-3'; *Xba*I site underlined) Tm^1 = 50 ºC
- **Primer BW:** PALIPBW (5'-GGA TCT AGA ACT GTT CGT TGT CAC C-3'; *Pst*I site underlined) Tm^1 = 53 ºC
- **Reaction :** amplification of *P. acnes gehA* gene including the upstream (76 bp) and downstream (84 bp) regions, Tm^2 = 50 ºC
- **Reference:** this study

C. OTHER AMPLIFICATIONS

pBR322 primers (Chapter 1□

- **Primer FW:** FWL37E (5'-GGA TGC TGT AGG CAT AGG C-3') Tm^1 = 58.1 ºC
- **Primer BW:** Primer B (5'-CAT CAA CGC ATA TAG CGC TAG C-3'; *Nhe*I site underlined) Tm^1 = 60 ºC
- **Reaction :** amplification of inserts cloned into *Eco*RV site of pBR322, Tm^2 = 50 ºC
- **Reference:** Prim *et al*., 2002

pUC19 primers (Chapter 1, 2 and 5□

- **Primer FW:** FWEA1 (5'-GAA AGG GGG ATG TGC TG-3') Tm^1 = 50 ºC
- **Primer BW:** BKEA1 (5'-GCT CGT ATG TTG TGT GG-3') Tm^1 = 48 ºC
- **Reaction :** amplification of inserts cloned into pUC19 polylinker, Tm^2 = 50 ºC
- **Reference:** Prim *et al*., 2001

Universal 16S rDNA primers (Chapter 2□

- **Primer FW:** Ty04F (5'-ATG GAG AGT TTG ATC CTG-3') Tm^1 = 54.6 ºC
- **Primer BW:** Ty06R (5'-TAC CTT GTT ACG ACT T-3') Tm^1 = 44.3 ºC
- **Reaction :** amplification of almost whole 16S rDNA sequence (~1453 bp), Tm^2 = 50 ºC
- **Reference:** Baker *et al*., 2003

♦ **Notes:** ● FW: forward; BW: backward.
● Tm^1: Theoretical melting temperature (at 50 mM Na^+) of each primer.
● Tm^2: Melting temperature at which the amplification reaction was performed.
● Primers were purchased from GIBCO BRL Custom Primers.

Table M.8 DNA amplification conditions.

***Taq* polymerase**

Reaction mixture composition

- 2 U *Taq* polymerase
- 5 µl 10× polymerase buffer
- 1.25 mM $MgCl_2$
- 250 µM of each dNTP
- 12 pmols of each primer (FW and BW)
- 1−100 ng template DNA (or 5 µl of 3 colonies suspended in 20 µl bidistilled water)
- Bidistilled water up to 50 µl

Amplification program

- 1 Cycle:
  - 5 min at 95 ºC
  - 30 s at Tm^2 + 2 ºC
  - 1−2 min at 72 ºC
- 29−35 Cycles:
  - 30 s at 96 ºC
  - 30 s at Tm^2
  - 1−2 min at 72 ºC
- Hold at 4 ºC

***Pfu* polymerase**

Reaction mixture composition

- 1 U *Pfu* polymerase
- 5 µl 10× polymerase buffer
- 250 µM of each dNTP
- 12 pmols of each primer (FW and BW)
- 1−100 ng template DNA (or 5 µl of 3 colonies suspended in 20 µl bidistilled water)
- Bidistilled water up to 50 µl

Amplification program

- 1 Cycle:
  - 5 min at 95 ºC
  - 30 s at Tm^2 + 2 ºC
  - 4 min at 72 ºC
- 29−35 Cycles:
  - 30 s at 96 ºC
  - 30 s at Tm^2
  - 4 min at 72 ºC
- Hold at 4 ºC

♦ **Note:** amplification temperature Tm^2 depended on the primers used (see Table M.7).

3.6 DNA SEQUENCING

Both DNA chains of purified PCR products or plasmids were sequenced using a *BigDye*® Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems) and the analytical system CE™ 8000 (Beckman-Coulter) available at the Serveis Científico Tècnics of the Universitat de Barcelona. This method is based on the Sanger dideoxy-mediated chain termination method (Sanger *et al*., 1977), and consists in the synthesis of DNA molecules in the presence of dideoxynucleotides (ddNTPs) that terminate the DNA synthesis and which are labelled with different fluorescent markers. The composition of the reaction mixture and the program used for DNA sequencing, according to the manufacturer's specifications, are detailed in Table M.9. DNA synthesis reaction was carried out in a GeneAMP PCR System 2400 thermocycler (Perkin Elmer). Amplified DNA was purified by a modified protocol of ethanol precipitation following the Serveis Científico Tècnics specifications, and then was analyzed by the CE™ 8000 analytical system.

Table M.9 DNA sequencing conditions.

Reaction mixture composition

- 4 µl *BigDye*® Terminator v. 3.1 Cycle ready reaction premix (contains thermostable polymerase, buffer, dNTPs and ddNTPs)
- 2 µl additional *BigDye*® buffer (10×)
- 3−300 ng DNA template
- 3.2 pmols primer (FW or BW)
- Bidistilled water up to 20 µl

Sequencing program

- 1 Cycle:
  - 3 min at 94 ºC
- 25 Cycles:
  - 10 s at 96 ºC
  - 5 s at primer Tm (see Tm^1 in Table M.7)
  - 4 min at 60 ºC
- Hold at 4 ºC

3.7 COMPUTER ANALYSIS OF DNA/PROTEIN SEQUENCES

Percent similarity of the resulting DNA or protein sequences was analyzed through BLAST (Altschul *et al*., 1997; http://www.ncbi.nlm.nih.gov/blast/), and sequence alignments were performed using ClustalW Multalign on-line software (Higgins *et al.,* 1994; http://www.ebi.ac.uk/clustalw/).

Phylogenetic trees based on 16S rDNA sequences were carried out using matrices of evolutionary distances computed from sequence alignments performed using the MEGA2 software (Sudhir *et al*., 2001). Distance matrices were obtained according to the Neighbor-Joining algorithm and using the Jukes and Cantor model (Jukes & Cantor, 1966). To validate the reproducibility of the branching pattern, a bootstrap analysis was performed.

The BioEdit Sequence Alignment Editor v. 5.0.9 (Hall, 1999) was used for several analyses of the DNA sequences obtained, including restriction pattern determination and identification of open reading frames (ORFs).

The ExPASy proteomics server (http://www.expasy.org) was used to analyze the physico-chemical parameters (ProtParam tool: Kyte & Doolittle, 1982; http://us.expasy.org/tools/protparam.html) and the presence of defined protein regions (ScanProsite: http://www.expasy.org/prosite) in the deduced amino acid sequences.

Superfamily assignments and analysis of the structural domains of the amino acid sequences were performed using Prodom (Servant *et al*., 2002; http://prodes.toulouse.inra.fr/prodom/current/html/home.php) and Search Pfam (Bateman *et al*., 2004; http://www.sanger.ac.uk/Software/Pfam/search.html).

Identification of putative signal peptides was performed through SignalP v. 2.0 software (Nielsen *et al*., 1997; http://www.cbs.dtu.dk/services/SignalP). Prediction of transmembrane regions was analyzed with DAS–Transmembrane prediction server (Cserzo *et al*., 1997; http://mendel.imp.univie.ac.at/sat/DAS/DAS.html), and prediction of protein subcellular localization was performed using PSORTb v. 2.0 for bacterial sequences (Gardy *et al*., 2004; http://www.psort.org/).

Secondary structure prediction was performed using the PSIPRED protein structure prediction server (McGuffin *et al*., 2000; 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/). Automated comparative three-dimensional modelling of cloned proteins was obtained from Swiss Model server (Peitsch, 1995; Guex & Peitsch, 1997; Schwede *et al*., 2003; http://www.expasy.org/Swissmod/SWISS-MODEL.html), using the Swiss-PdbViewer v. 3.7 software (http://www.expasy.org/spdbv/) to visualize the protein models.

3.8 ENZYMATIC TREATMENT OF DNA

3.8.1 Digestion with restriction enzymes

Restriction nucleases and their corresponding buffers were obtained from Roche or Bio-Labs and used according to the manufacturer's specifications. Double digestions were carried out using the most suitable buffer for both enzymes. However, when the enzymes were low- or non-compatible, double digestions were performed sequentially including a cleaning step by ethanol precipitation between first and second digestion.

Digestion reactions were finalized following the manufacturer's specifications (usually by incubation for 20 min at 65−80 ºC), and DNA was re-purified by phenol:chloroform treatment and ethanol precipitation.

3.8.2 RNAse treatment

When necessary (visualization of DNA in agarose gels, etc), samples containing RNA such as digestions of plasmids isolated by the LiCl minipreparation method were incubated for 5 min at RT with RNAse A (final concentration: 50 μg ml^{-1}) to remove the RNA.

3.8.3 Alkaline phosphatase treatment

The 5' ends of plasmids linearized using restriction enzymes were dephosphorilated using alkaline phosphatase in order to avoid plasmid re-ligation. The reaction was performed according to the manufacturer's specifications: addition of alkaline phosphatase to purified DNA, incubation for 1 h at 37 ºC, and finalization of the reaction by 10-min incubation at 65 ºC. Finally, DNA was re-purified by phenol:chloroform treatment and ethanol precipitation.

3.8.4 Ligation of DNA molecules

Ligation reactions were carried out using T4 DNA ligase according to the manufacturer's specifications. Ligation of molecules with cohesive termini was performed O/N at 16 ºC using an insert:plasmid ratio of 3:1. Blunt-ended DNA was ligated O/N at 4 ºC using an insert:plasmid ratio of 6:1. Ligation of digested genomic DNA for Inverse PCR assays was carried out O/N at 16 ºC using high DNA dilutions to favour intramolecular re-ligation. Ligation reactions were stopped by incubation for 10 min at 65 ºC, and DNA was re-concentrated and re-purified by ethanol precipitation.

3.9 TRANSFORMATION OF *E. coli* WITH EXOGENOUS DNA

Transformation of *E. coli* strains with exogenous DNA was performed essentially by the method of Cohen *et al*. (1972).

Preparation of *E. coli* competent cells: *E. coli* cells were grown in LB supplemented with the corresponding antibiotics, when it was necessary. The culture was maintained under agitation at 37 ºC until $A_{600\ nm}$ was 0.4−0.6 (mid exponential phase). Then, cells were harvested by centrifugation at 5000 ×g for 5 min at 4 ºC, and suspended in ½ volumes of 50 mM $CaCl_2$ at 4 ºC. After 10-min

incubation in ice, cell-harvesting and suspension steps were repeated using $^{1}/_{10}$ volumes of 50 mM $CaCl_2$ at 4 ºC. The resulting competent cells were maintained for 1−24 h in ice before being used. For longer period storage, 20% (v/v) glycerol was added to competent cells, which were kept at −80 ºC.

Transformation of competent cells: 100 µl of competent cells were mixed with the desired exogenous DNAs (final concentration: 2−10 ng μl^{-1}). The resulting mixtures were incubated for 45 min at 4 ºC. Then, the samples were heated for 2 min at 42 ºC and transferred to ice. After addition of 0.9 ml LB, the samples were incubated under agitation for 1 h at 37 ºC to allow the expression of the plasmid genes involved in resistance to antibiotics. After that, cells were harvested by centrifugation at 13000 ×g for 2 min at RT, and suspended in 300 µl of fresh LB medium. Then, 25−100 µl of transformed cells were spread on LB agar plates supplemented with the suitable antibiotics and reagents (IPTG, X-gal, etc) for the selection of the transformed/recombinant cells. Finally, these plates were incubated O/N at 37 ºC.

3.10 HYBRIDIZATION OF DNA MOLECULES

3.10.1 Preparation of labelled DNA probes

Preparation of digoxigenin-labelled probes for the consensus region of *B. subtilis lipA*–related lipases (Chapter 1) was performed by amplification of the mentioned DNA region adding 5 µl of DIG DNA 10× labelling mixture to the typical *Taq* reaction mixture (see Table M.8). The resulting digoxigenin-labelled probes were recovered by ethanol precipitation.

3.10.2 Dot blot technique

Dot blot was performed as described by Sambrook *et al*. (1989). The composition of the solutions used is shown in Table M.10. Nylon membranes (Roche) used in Dot blot assays were first washed with distilled water and then equilibrated in denaturing buffer. Once equilibrated, 5 µl of each DNA sample were loaded on the membranes, which were placed on top of a filter paper equilibrated with denaturing buffer, and incubated for 5 min. After the denaturation process, DNA was fixed to the nylon membranes by irradiating each membrane face with UV light (310 nm) for 90 s. Then, the membranes were equilibrated in neutralizing buffer before being dried for 1 h at 80 ºC. Further probe hybridization and detection was performed as is described in General Materials and Methods 3.10.4.

3.10.3 Southern blot technique

Southern blot was performed as described by Sambrook *et al*. (1989). The composition of the solutions used is shown in Table M.10. DNAs to be hybridized were digested, analyzed in agarose gels, stained and visualized. Then, agarose gels were treated with 0.25 M NaOH for 15 min. After that, gels, nylon membranes and filter paper were equilibrated in transfer buffer. DNA transfer to nylon membranes was carried out for 1 h at 60 volts using a Mini-Protean II (Bio-Rad) equipment. Membranes were then dried for 30 min at RT, and DNA was fixed to nylon membranes by irradiating each membrane face with UV light (310 nm) for 90 s. Further probe hybridization and detection was performed as is described in General Materials and Methods 3.10.4.

3.10.4 DNA hybridization and probe detection

Hybridization and detection of dried Dot/Southern blot DNA-containing membranes was performed essentially as described (Sambrook *et al*., 1989) using the solutions detailed in Table M.10.

Table M.10 Solutions used for Dot blot and Southern blot assays.

MEMBRANE PREPARATION

Denaturing buffer (Dot blot□

- 1.5 M NaCl
- 0.5 M NaOH

Neutralizing buffer (Dot blot□

- 0.5 M Tris–HCl (pH 7.2)
- 1.5 M NaCl
- 1 mM EDTA (pH 8)

Transfer buffer (Southern blot□

- 40 mM Tris–HCl (pH 8.3)
- 40 mM Boric acid
- 1 mM EDTA (pH 8)

HYBRIDIZATION

SSC 10×

- 0.15 M sodium citrate
- 1.5 M NaCl
- Adjust to pH 7

Hybridization solution

- SSC 5×
- 5 % (w/v) blocking reagent
- 0.02 % (w/v) SDS
- 0.1 % (w/v) sodium laurylsarcosinate

PROBE DETECTION

Buffer 1

- 0.1 M Tris–HCl (pH 7.5)
- 0.15 M NaCl

Buffer 2

- 0.5 % (w/v) blocking reagent prepared in Buffer 1

Buffer 3

- 0.1 M Tris–HCl (pH 9.5)
- 0.15 M NaCl
- 50 mM $MgCl_2$

Buffer 4

- 0.1 M Tris–HCl (pH 8)
- 1 mM EDTA (pH 8)

Chromatic solution

- 10 ml Buffer 3
- 200 µl NBT/BCIP stock solution (Roche)

Membranes were first prehybridized in hybridization solution for 3 h. After that, this solution was removed and replaced by hybridization solution at 60 ºC containing 0.5 µg ml^{-1} digoxigenin-labelled probe previously denatured for 5 min at 100 ºC and

subsequently cooled in ice. Hybridization was performed O/N at 60 ºC, and was followed by two washing-steps for 5 min using SSC 2× solution (at RT) containing 0.1 % (w/v) SDS, and two washing-steps for 15 min using SSC 0.1× solution (at 60 ºC) containing 0.1 % (w/v) SDS.

Detection of the labelled-probe–hybridized DNA fragments was carried out using an anti-digoxigenin antibody linked to alkaline phosphatase. Hybridized membranes were washed for 1 min in buffer 1, incubated for 30 min in buffer 2, and washed again in buffer 1 for 1 min to remove all unbound probe molecules. After that, membranes were incubated for 30 min with the antibody (diluted 1:5000 in buffer 1), washed twice for 15 min in buffer 1 to eliminate the unbound antibody molecules, and subsequently equilibrated in buffer 3. Finally, the probe-hybridized DNA molecules were detected by incubation of the membranes with the chromatic solution in the absence of light, until the appearance of the precipitated signal. Then, the reaction was stopped using buffer 4 and, after buffer elimination, membranes were dried at RT.

4 PROTEIN TECHNIQUES

4.1 CULTURE FRACTIONATION

4.1.1 Preparation of cell extracts and culture supernatants

Crude cell extracts and culture supernatants of lipolytic strains and *E. coli* recombinant clones were prepared essentially as described before (Prim, 2002). Briefly, liquid cultures were centrifuged at 4000 ×g for 10 minutes at 4 ºC. The supernatants were recovered and stored at 4 ºC, whereas the pellets were suspended in 50 mM phosphate buffer (pH 7), lysed, and cleared by centrifugation at 10000 ×g for 10 minutes at 4 ºC. The resulting cleared cell lysates were recovered and considered as the cytoplasm fractions (cell extracts), whereas the final pellets were considered as the cell-membrane fractions. Membrane fractions were also suspended in 50 mM phosphate buffer (pH 7), when it was necessary.

For low culture-volumes (less than 0.5 l) the lysis process was performed by sonication: 2 cycles (separated by 20 s of repose to avoid excessive heating) of 0.9 s pulses at 50 watts for 2 min in a Labsonic 1510 sonicator (B. Braun). Higher culture volumes were lysed by 3 pressure-cycles at 1000 PSIG using a French Pressure Cell Press SLM (AMINCO).

Cell extracts, membranes and culture supernatants were always manipulated and maintained at 4 ºC. Long-time storage was performed at −20 ºC.

4.1.2 Protein concentration

Protein solutions (supernatants, purified–protein fractions, etc) were concentrated by centrifugation at 5000 ×g (4 ºC) using ultrafiltration Centricon™–10 filters (Amicon®) of a molecular weight exclusion size of 10 kDa.

4.2 DETERMINATION OF PROTEIN CONCENTRATION

Protein concentration of the samples was determined using the Bio-Rad Protein Assay (Bio-Rad), which is based on the Bradford method (Bradford, 1976). Bovine serum albumin solutions at concentrations from 0 to 1 mg ml^{-1} were used as standard.

4.3 PROTEIN PURIFICATION

4.3.1 Purification by the His-tag system

Purification by His-tag based affinity chromatography was performed essentially as described by pET28a manufacturer's (Novagen). The composition of the specific solutions used is detailed in Table M.11.

4.3.1.1 Sample preparation

This method required the previous construction of pET28a-EstV plasmid (Table M.3; Chapter 5), and its transformation in *E. coli* BL21(DE3) (see Table M.1). In this plasmid, the lipase gene of interest was cloned in the following configuration: T7 promoter/*lac* operator/N-terminal 6-His tag/thrombin cleavage site/lipase gene/T7 terminator. BL21(DE3), which contains T7 RNA polymerase gene regulated by the *lac* promoter (λDE3 lysogen), was used as host strain. All the system is regulated by IPTG induction (see Figure M.4).

E. coli BL21(DE3)–pET28a-EstV was grown in 1 litre of LB Kan at 37 ºC (under agitation). When the $A_{600\ nm}$ was 0.5−0.6, 1 mM IPTG was added, and the culture was incubated for 3 h before harvesting cells by centrifugation at 11000 ×g for 10 min at 4 ºC. Cell extract was obtained by recovering the supernatant resulting from suspending cells in 20 ml lysis buffer, pressure lysis, and subsequent centrifugation at 16000 ×g for 30 min at 4 ºC.

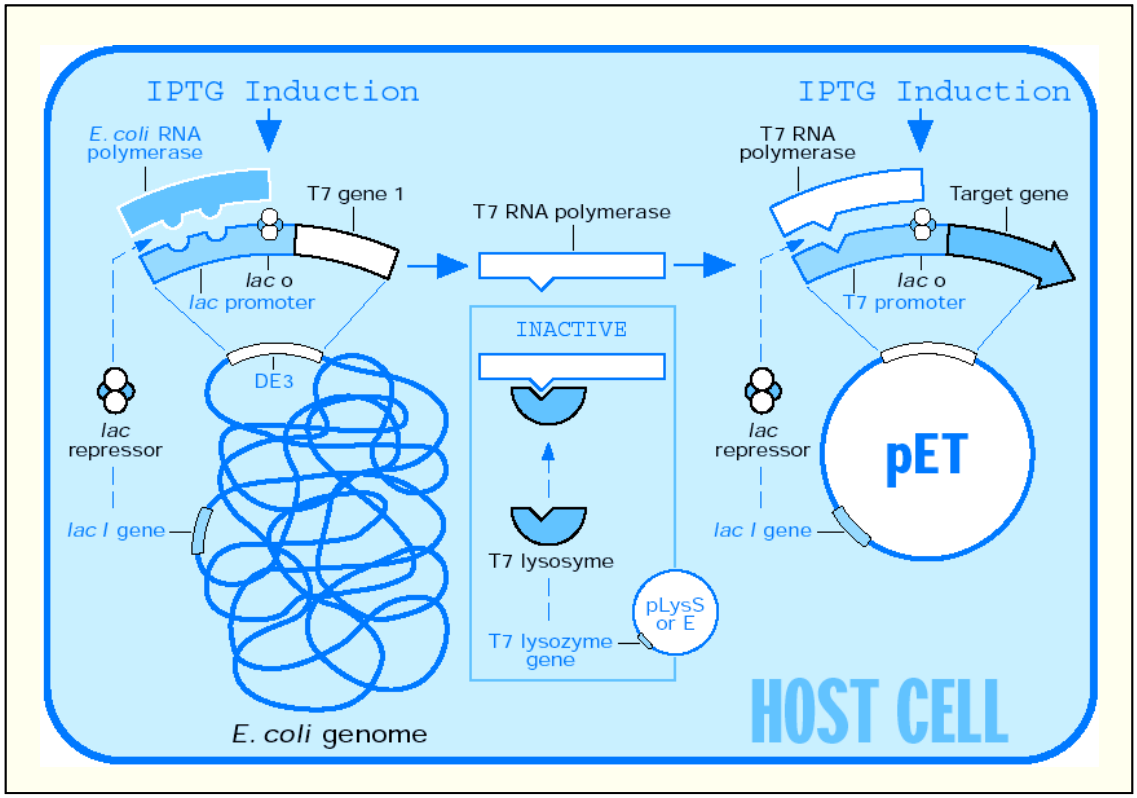

Figure M.4 Control elements of the pET-28a–*E. coli* BL21 (DE3□

4.3.1.2 Purification

Purification of His-tag proteins from cell extracts was preformed by affinity chromatography using NTA-Ni (nitrilotriacetic-nickel acid) agarose resin, which is based on the affinity of biomolecules with six consecutive histidines to nickel. All the manipulations and centrifugations were performed at 4 ºC, and all the solutions were pre-cooled at 4 ºC before being used. First, 250 µl of NTA-Ni resin were added to 5 ml of cell extract. After vertical agitation for 2 h, the mixture was centrifuged at 13000 ×g for 10 min and the supernatant was discarded. The pellet (NTA-Ni resin containing the His-tag protein) was washed 9 times by suspension in washing buffer–20 mM imidazole, 1-min centrifugation at 13000 ×g, and supernatant removal. After that, washing was repeated twice using washing buffer–50 mM imidazole, and 1 more time using washing buffer–200 mM imidazole. Finally, elution was performed for 3 times by sample suspension in elution buffer, 1-min centrifugation at 13000 ×g, and supernatant recovery. The purified lipase from the corresponding supernatants was analyzed by

SDS-PAGE and zymogram for molecular mass and activity detection (General Materials and Methods 4.4.1 and 5.2, respectively).

The His-tail of purified proteins was removed using the Thrombin Cleavage Capture Kit from Novagen. The elimination reaction (composition shown in Table M.11) was incubated for 16 h at 20 ºC, and the biotinylated thrombin was then eliminated using 16 µl of streptavidine agarose per thrombin Unit. The binding reaction between biotinylated thrombin and streptavidine agarose was incubated by vertical agitation for 30 min at RT. Finally, this mixture was filtrated and the purified protein without His-tail, and without thrombin, was recovered and analyzed by SDS-PAGE and zymogram.

Table M.11 Solutions for protein purification by the His-tag system.

Lysis buffer

- 0.3 M NaCl
- 50 mM NaH_2PO_4
- 10 mM imidazole
- Adjust to pH 8

Elution buffer

- 0.3 M NaCl
- 50 mM NaH_2PO_4
- 250 mM imidazole
- Adjust to pH 8

Washing buffers

- 0.3 M NaCl
- 50 mM NaH_2PO_4
- 20, 50 or 200 mM imidazole
- Adjust to pH 8

His-tag elimination reaction

- 500 µl thrombin buffer 10×
- 1 mg purified protein
- 2 µl biotinylated thrombin
- Bidistilled water up to 5 ml

4.3.2 Purification by FPLC

Purification by FPLC (Fast Protein Liquid Chromatography) was performed following the manufacturer's instructions in a chromatographic system that consisted of an AKTA FPLC (Amersham Biosciences) controlled by the UNICORN v. 4.12 software package for Windows®.

4.3.2.1 Sample preparation

E. coli DH5α–pUC-EstV (Table M.1; Chapter 5) was grown in 2 litres of LB Ap at 37 ºC (under agitation). When the $A_{600\ nm}$ was 0.5−0.6, 1 mM IPTG was added, and the culture was incubated O/N before harvesting cells by centrifugation at 11000 ×g for 10 min at 4 ºC. Cell extract was obtained by recovering the supernatant resulting from suspending cells in 20 ml of 50 mM phosphate buffer (pH 7), pressure lysis, and subsequent centrifugation at 16000 ×g for 10 min at 4 ºC.

Next step consisted in the elimination of nucleic acids and small membrane fragments from cell extracts by adding 2.5 mg ml^{-1} streptomycin sulphate. Addition was carried out slowly and under agitation, and was followed by 2 h-incubation at 4 ºC. After that, cell extracts were centrifuged at 150000 ×g for 50 min at 6 ºC, and the supernatants recovered.

A number of undesired proteins from cell extracts were firstly eliminated by salting out by slow addition of 10% (w/v) ammonium sulphate to cell extracts under gentle agitation. After 10-min incubation at 4 ºC and subsequent centrifugation at 16000 ×g for 1 h at 4 ºC, the supernatants were recovered. Precipitation was then repeated adding ammonium sulphate up to a final concentration of 40% (w/v), although in this case centrifugation was followed by supernatant elimination and pellet recovery. The pellets were suspended in 5 ml of 50 mM phosphate buffer (pH 7) and centrifuged again at 11000 ×g for 10 min at 4 ºC. Finally, the supernatants were recovered, ready to be injected into the FPLC.

4.3.2.2 Purification

Purification of concentrated protein suspensions was performed at RT using three different separation columns in the previously mentioned AKTA FPLC chromatographic system.

First, 2 ml aliquots of protein suspensions were loaded into 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) containing 0.02% (w/v) sodium azide. Protein separation by gel filtration (by size) was monitored at a wavelength of 280 nm, and the eluated protein fractions were recovered in fractions of 2 ml. Lipase-containing fractions were detected by colorimetric microassay (General Materials and Methods 5.3.2), and were analyzed by SDS-PAGE and zymogram (General Materials and Methods 4.4.1 and 5.2, respectively).

The fraction showing the highest lipase activity was concentrated, and proteins were subsequently separated by ion exchange (by isoelectric point -pI-). Aliquots of 1 ml were injected to 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 in 50 mM glycine–NaOH buffer (pH 10; 1 point over EstV pI to have an electrically negative-charged protein able to bind to the column) containing 0.02% (w/v) sodium azide. Protein separation by ion exchange was performed with a linear gradient of NaCl (0−1 M) in 50 mM glycine–NaOH buffer (pH 10) containing 0.02% (w/v) sodium azide. The eluated proteins were recovered in fractions of 1 ml. Lipase-containing fractions were then detected by colorimetric microassay, analyzed by SDS-PAGE, and concentrated.

The resulting protein solution was re-purified by repeating the gel filtration separation using a different column. In this case, aliquots of 0.5 ml were injected 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) containing 0.02% (w/v) sodium azide. The eluated proteins were recovered in fractions of 1 ml, and those containing the purified lipase were detected by colorimetric microassay and analyzed by SDS-PAGE and zymogram. Highly purified lipase was obtained at this step. However, a second ion exchange separation under the same conditions as the previous one was performed for assays requiring a completely pure enzyme (N-terminal sequencing, etc).

4.4 PROTEIN ELECTROPHORESIS

4.4.1 SDS-PAGE electrophoresis

Proteins were separated by molecular weight (MW) using polyacrylamide gels containing SDS as denaturing agent (SDS-PAGE gels) as is described (Laemmli, 1970). The composition of the solutions and gels used is detailed in Table M.12

Loading buffer 3× was added to the samples, which were usually incubated for 5 min at 100 ºC, and maintained in ice before being loaded into SDS-PAGE gels. Electrophoresis was carried out at 75 volts through the stacking gel, and at 100 volts through the resolving gel, using a Mini-protean-II gel tray (Bio-Rad) filled with running buffer. Once the electrophoresis ended, gels were stained for 1 h in Coomassie solution, and were subsequently faded with 1 % (v/v) acetic acid until clear visualization of protein bands was achieved. When zymogram analysis was performed (see General Materials and Methods 5.2) it was carried out before the Coomassie staining.

The MW of sample proteins was determined by comparison of their migration in SDS-PAGE gels with that of a broad-range MW marker (SDS-PAGE standards Broad Range Molecular Weight from Bio-Rad; 201.66−6.67 kDa;). The MW (kDa) of marker proteins was: 201.66 (myosin), 116.43 (β-galactosidase), 93.59 (phosphorilase B), 66.2 (bovine serum albumin), 53.62 (ovoalbumin), 37.24 (carbonic anhydrase), 29.05 (soybean trypsin inhibitor), 19.65 (lysozyme) and apoprotinin (6.67).

Table M.12 Gel composition and solutions used for SDS-PAGE gels.

Acrylamide-bisacrylamide solution

- 30 g of acrylamide
- 0.8 g of bisacrylamide
- Distilled water up to 100 ml
- Filtration + storing at 4 ºC (light-protected)

Stacking buffer

- 0.5 M Tris–HCl (pH 6.8)
- 0.4% (w/v) SDS

Resolving buffer

- 1.5 M Tris–HCl (pH 8.8)
- 0.4% (w/v) SDS

Running buffer (pH 8.3)

- 25 mM Tris
- 192 mM glycine
- 1% (w/v) SDS

Loading buffer 3×

- 62.5 mM Tris–HCl (pH 6.8)
- 10% (v/v) glycerol
- 2.3% (w/v) SDS
- 2.5% (v/v) β-mercaptoethanol
- 0.02% (w/v) bromophenol blue

Stacking gel composition (5%)

- 0.42 ml of acrylamide-bisacrylamide solution
- 0.625 ml stacking buffer
- 1.4 ml of distilled water
- 5 µl of TEMED
- 17.5 µl of 10% (w/v) APS

Resolving gel composition (12%)

- 2.4 ml of acrylamide-bisacrylamide solution
- 1.56 ml resolving buffer
- 2 ml of distilled water
- 5 µl of TEMED
- 30 µl of 10% (w/v) APS

Coomassie solution

- 0.05% (w/v) Brilliant Blue Coomassie® R250
- 10% (v/v) acetic acid
- 25% (v/v) isopropanol

4.4.2 Native-PAGE electrophoresis

Electrophoresis in native-PAGE gels for separation of non-denatured proteins by electric charge and conformation was carried out as described by Hames & Rickwood (1981). Assays were performed using the same conditions and solutions as for SDS-PAGE electrophoresis (see General Materials and Methods 4.4.1), but preparing all solutions without SDS (non-denaturing conditions).

4.5 N-TERMINAL SEQUENCING OF PROTEINS

Sequencing of the N-terminal end of proteins was performed by the method of Edman degradation according to the specifications of Packman (1993).

First step consisted in a SDS-PAGE of the protein sample performed as is described (see General Materials and Methods 4.4.1), with the following modifications: (1) acrylamide-bisacrylamide solution was deionized for 2 h at 4 ºC by the addition of 5% (w/v) Grade Mixed Bed Resin from Bio-Rad, after that, the solution was filtrated before being used to the preparation of the gels, (2) 2 mM thioglycolic acid was added to the running buffer, (3) the gels were pre-run without samples for 20 min at 130 volts to remove contaminants and ions from gels, (4) the samples were run through the stacking gel at 40 volts, (5) the protein marker used was the pre-stained version of the previously described SDS-PAGE standards Broad Range from Bio-Rad.

After electrophoresis gels were treated for 5 min in methanol, and for 5 min in transfer buffer (48 mM Tris, 39 mM glycine, 20% (v/v) methanol and 0.02% (w/v) SDS; pH 8.3). Then, proteins were transferred to a PVDF membrane (Roche) for 1 h at 15 volts using a Trans-Blot Semi-Dry (Bio-Rad) filled with transfer buffer. After that, the membrane was 3-times washed for 10 min in bidistilled water, stained for 5 min in staining solution (methanol:distilled water at 50:50, by vol, plus 0.01% (w/v) Brilliant Blue Coomassie® R250), and was finally faded in methanol:acetic acid:distilled water (50:10:40, by volume). Then, the membrane was dried at RT, and the bands of interest were cut out and frozen at −20 ºC. Cut bands were sequenced at the Servei de Seqüenciació de Proteïnes of the Universitat Autònoma de Barcelona using the analytical system Procise 492 (Applied Biosystems).

4.6 ISOELECTRIC FOCUSING

The isoelectric point (pI) of proteins was determined by isoelectric focusing (IEF). The system is based on protein migration through a pH-gradient gel under increasing voltage conditions. When protein reaches a gel point with a pH equal to its own pI, loses its electric charge and its migration ends.

IEF was carried out by a PhastSystem unit (Pharmacia Biotech), using PhastGel® IEF gels (pH range: 3−9; Pharmacia Biotech) or Servalyt® Precotes® gels (pH range: 3−10; SERVA), according to manufacturer's specifications.

Protein separation was followed first by zymogram analysis (see General Materials and Methods 5.2), and protein staining later. Proteins were stained using Coomassie staining according to the modifications described by Blanco *et al*. (1996). Briefly, proteins were fixed for 15 min in 20% (v/v) trichloroacetic acid. Then, the gels were washed for 5 min in washing solution (methanol:acetic acid:distilled water at 30:10:60, by volume), subsequently stained for 15 min in staining solution (washing solution plus 0.02% (w/v) Brilliant Blue Coomassie® R250 and 0.1% (w/v) $CuSO_4$), and finally faded in washing solution until protein bands were visualized.

The pI of sample proteins was determined by comparison of their migration in IEF gels with that of a pI marker (Broad pI kit Marker: pI range of 3.5−9.3; Pharmacia). The pI of the marker proteins used was: 9.30 (trypsinogen), 8.65 (lectine), 8.45 (lectine), 8.15 (lectine), 7.35 (horse myosin), 6.85 (horse myosin), 6.55 (carbonic anhydrase A), 5.85 (carbonic anhydrase B), 5.20 (β-lactoglobuline A), 4.55 (trypsin inhibitor), 3.50 (amyloglucosidase).

5 ENZYMATIC ASSAYS

5.1 PLATE ASSAYS

5.1.1 Plate assays using lipid substrates

The lipolytic activity of strains and recombinant clones was detected by means of a direct test based on streaking these strains on CeNAN agar plates supplemented with tributyrin, triolein or olive oil (Table M.2), and subsequent incubation at the optimum temperature of the microorganism (Table M.1). True lipase activity was detected after growth in emulsified CeNAN-olive oil/triolein agar, due to the formation of complexes between released long-chain fatty acids and Rhodamine B. These complexes were detected by the presence of pink/orange-coloured colonies and by fluorescence emission under UV light irradiation (Kouker & Jaeger, 1987). Carboxylesterase activity was detected by hydrolysis haloes visible around the colonies grown on CeNAN–tributyrin agar plates (Prim *et al*., 2000).

5.1.2 Plate assays using polysaccharides

Cellulase, xylanase and pectinase activities of isolated strains (Chapter 2) were screened by streaking these strains on agar plates supplemented with the desired substrate (Table M.2): carboxymethyl cellulose, xylan, and polygalacturonic acid (PGA), respectively. Plate incubations were performed at the isolation temperature of each strain. Xylanase activity was observed by direct appearance of hydrolysis areas surrounding the colonies after growth on LB-xylan plates (Blanco & Pastor, 1993). Cellulase activity was detected after growth on LB-carboxymethyl cellulose plates followed by 15 min-staining with 1% (w/v) Congo Red and subsequent washing with 1 M NaCl until the appearance of hydrolysis haloes around the streak (Blanco *et al*., 1998). Pectinase activity was detected after growth on Nutrient broth-PGA plates and

subsequent PGA-precipitation using 1 M HCl by the appearance of hydrolysis haloes surrounding the colonies (Soriano *et al*., 2000).

5.2 ZYMOGRAM ANALYSIS

Lipolytic activity of proteins separated by SDS-PAGE, native-PAGE or IEF gels was detected by zymogram analysis before Coomassie staining of the gels. Zymograms were performed as previously described (Díaz *et al*., 1999; Prim *et al*., 2003). After the run, gels were soaked for 30 min at RT in 2.5% (v/v) Triton X-100, washed twice in 50 mM phosphate buffer (pH 7) for 20 min (at RT), and covered by a solution of 100 μM MUF-butyrate or 400 μM MUF-oleate in the same buffer. Activity bands resulting from MUF releasing became visible in a short time after UV illumination, although low active samples (and almost all gels treated with MUF-oleate) required longer incubation periods at the optimum temperature of the enzyme.

5.3 ASSAYS USING *p*-NP-DERIVATIVES

5.3.1 Determination of lipase activity by colorimetric assay

Lipolytic activity determination was performed by a previously described colorimetric assay (Prim *et al*., 2000; Prim *et al*., 2003).

Stock solutions of *p*-NP-derivatives were freshly prepared in isopropanol at a concentration of 0.3% (w/v or v/v depending on the substrate) except for *p*-NP palmitate and *p*-NP stearate (prepared at 0.15%, w/v). These solutions were emulsified by 3-min sonication pulses of 0.3 s at 50 watts using a Labsonic 1510 sonicator (B. Braun).

Then, 450 µl of a 1:10 dilution of the substrate stock solution in solution B (Table M.13) were preincubated for 5 min at the assay temperature before adding 50 µl of lipase sample (cell extracts). This mixture was incubated at the assay temperature for 15 min, and the reaction was terminated by addition of 35 µl of 0.1 M Na_2CO_3 and 535 µl of bidistilled water.

Table M.13 Composition of solution B.

Solution B
• 50 mM phosphate buffer (pH 7)
• 0.1% (w/v) arabic gum
• 0.4% (v/v) Triton X-100

Released *p*-nitrophenol was immediately determined by measuring the absorbance at λ = 405 nm ($A_{405\ nm}$) in a Spectophotometer Du® (Beckman Coulter). Proper blanks were used to subtract the absorbance corresponding to the reaction mixture other than that produced by the specific hydrolysis of *p*-NP-derivatives. One unit of activity was defined as the amount of enzyme that released 1 µmol of *p*-nitrophenol per minute under the assay conditions described.

5.3.2 Determination of lipase activity by a new colorimetric microassay

The previously described colorimetric assay (see General Materials and Methods 5.3.1) was adapted and simplified to obtain a fast, simple and more sensitive colorimetric microassay suitable for high-throughput evaluation of lipase activity and lipase inhibition (see Chapter 3 for more details).

The substrate (usually *p*-NP laurate - *p*-NPL -, but also other *p*-NP-derivatives) was dissolved at 20 mM in isopropanol by continuous sonication for 3 minutes in a Ultrasons sonicator bath (Selecta). A 1:10 (v/v) dilution in phosphate–Triton buffer (55.56 mM phosphate buffer (pH 7) containing 1.33% (v/v) Triton X-100) was subsequently prepared by gentle agitation until an optically clear and stable emulsion was achieved. Then, 50 µl doses of this mixture were dispensed into a round-bottom 96-well microtiter plate and preincubated for 15 minutes at the assay temperature (usually 37 °C).

After substrate preincubation, 50 µl of each enzyme solution (0−5 mg ml^{-1}), prepared in 50 mM phosphate buffer (pH 7) and also preincubated for 15 minutes at the assay temperature, were added to the previous substrate mixture to obtain the final reaction mixture (100 µl containing: 1 mM *p*-NPL, 5% isopropanol, 0.6% Triton X-100, 50 mM phosphate buffer (pH 7) and 0−2.5 mg ml^{-1} of enzyme). The reaction mixture was then incubated for 15−30 additional minutes at the assay temperature and the absorbance at λ = 405 nm of each well was measured in a microtiter plate reader (BIO-RAD Model 3550) to determine the release of *p*-nitrophenol.

Proper blanks were performed to subtract the absorbance of the reaction mixture other than that produced by the specific hydrolysis of *p*-NP-derivatives. All assays were performed within the linear range of absorbance of the *p*-nitrophenol calibration curve, which was obtained under strict assay conditions to avoid the absorbance changes of *p*-nitrophenol at different pH-values (Iacazio *et al*., 2000). 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*-nitrophenol calibration curve. One unit of activity was defined as the enzymatic activity that released 1 µmol of *p*-nitrophenol per minute under the assay conditions.

5.4 ASSAYS USING MUF-DERIVATIVES

5.4.1 Detection of lipolytic activity by fluorimetric paper assay

Detection of lipolytic activity from purified lipases, cell extracts, or cell suspensions on 50 mM phosphate buffer (pH 7) was performed by the fluorimetric paper assay (Díaz *et al*., 1999). This method consisted in transferring a small aliquot (5 µl) of each sample onto a filter paper, followed by addition of 5µl of 25 mM MUF-butyrate (or 100 mM MUF-oleate) stock solution and subsequent UV illumination of the paper. Detection of lipolytic activity on MUF-butyrate was achieved in less than 1 min, while hydrolysis of MUF-oleate usually required longer incubation periods at RT.

5.4.2 Determination of lipolytic activity by fluorimetric liquid assay

5.4.2.1 Classical fluorimetric liquid assay

Fluorimetric assays using MUF-derivative substrates were performed as previously described (Prim *et al*., 2000; Prim *et al*., 2003), using a Hitachi F-2000 spectrofluorimeter (Hitachi).

Direct assays were performed at RT and as time–drive plots. The fluorescence emission caused by MUF release from fluorogenic substrates was measured at 400 volts using a λ_{ex} = 323 nm and a λ_{em} = 448 nm, established as the maximum under our assay conditions (Prim *et al*., 2000). The reaction mixture consisted of 1436 µl of solution B (Table M.14) to which 6 µl of MUF-derivative stock solution (usually MUF-butyrate) and 60 µl cell extract (at a proper concentration to obtain non-saturated linear plots) were added. Activity was determined by measuring the increase of fluorescence emission caused by the release of MUF during hydrolysis, according to previously generated MUF-standard plots. Activity was calculated after subtracting unspecific hydrolysis with proper blanks. One unit of activity was defined as the amount of enzyme required to release 1 µmol of MUF per minute under the conditions described.

Experiments requiring specific temperatures and incubation times (determination of optimum temperature and pH, and stability to different temperatures or pH values) were performed as previously described, but using end-point assays: solution B containing MUF-butyrate was preincubated at the assay temperature for 5 min before adding a proper enzyme solution. Then, the reaction mixtures were incubated for 15 min at the assay temperature before ending the reaction with 30 µl of 20% (v/v) HCl. After incubating the samples for 10 min at RT (to have a constant temperature during fluorescence detection) fluorescence emission was determined as described before.

5.4.2.1 New fluorimetric assay

The development of the new colorimetric microassay (see General Material and Methods 5.3.2 and Chapter 3) led to the development of an equivalent method for fluorescent assays replacing *p*-NP-derivatives by MUF-derivative substrates.

Reaction premix (55.56 mM phosphate buffer (pH 7) containing 1 mM MUF-butyrate and 0.7% (v/v) Triton X-100) and enzyme solution (prepared in 50 mM phosphate buffer (pH 7) at a proper concentration to have a linear assay) were preincubated for 5 min at the assay temperature. Then, 10 µl of enzyme solution were added to 90 µl of reaction premix to obtain the final reaction mixture (100 µl), which was incubated for 15 min at assay temperature before ending the reaction with 400 µl of stopping solution (30 µl of 20% (v/v) HCl plus 370 µl of distilled water).

Samples were then incubated for 10 min at RT, and subsequently analyzed in a Cary Eclipse spectrofluorimeter (Varyan). Analysis was performed at 400 volts using a λ_{ex} = 323 nm, a λ_{em} = 448 nm, a excitation slit of 20 nm, an emission slit of 10 nm, and an average time recording of 2 s, established as the optimum parameters under our assay conditions. Activity was determined, after subtracting unspecific hydrolysis with proper blanks, by comparison of the increase in fluorescence emission obtained with respect to that of the previously generated MUF-standard plots. One unit of activity was defined as the amount of enzyme required to release 1 µmol of MUF per minute under the conditions described.

5.5 DETERMINATION OF LIPASE ACTIVITY BY HPLC

Lipase assays by HPLC were performed as previously described (Grippa *et al*., 1999). Essentially, 2020 µl reaction mixtures containing 0.46 mM β-naphtyl laurate (β-NL), 1.25 % (v/v) acetone, 1 mM sodium taurocholate, 3.5 mM NaCl, 1.5 mM $CaCl_2$, 50 mM Tris–HCl buffer (pH 7.4) and 10 µg ml^{-1} of *Candida rugosa* lipase (CRL) were incubated for 30 minutes at 37 °C under gentle mixing. Then, β-naphtol (β-N) was extracted with 2 ml of ethyl acetate, and 500 µl of the organic phase were withdrawn, evaporated at RT 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. One unit of activity was defined as the amount of enzyme that released 1 μmol of β-N per minute under the assay conditions described.

5.6 BIOCHEMICAL CHARACTERIZATION OF LIPASES

5.6.1 Optimum temperature

Optimum temperature assays were performed by the previously described fluorimetric liquid assays (either the classical-endpoint assay or the new fluorimetric assay) using the following assay temperatures (ºC): 4, 20, 30, 35, 37, 40, 42, 45, 50, 55, 60, 70 and 80. Initially, reactions were performed in reaction mixtures containing phosphate buffer 50 mM (pH 7) (Prim *et al*., 2000). Once the optimum temperature was known, the same process was repeated at the optimum pH found after the optimum pH assays.

5.6.2 Optimum pH

Optimum pH assays were performed by the previously described fluorimetric liquid assays (either the classical-endpoint assay or the new fluorimetric assay) replacing the phosphate buffer (50 mM final concentration; pH 7) used in the standard reaction mixture by the following buffers (at a final concentration of 50 mM, and prepared as described by Gomori (1955)): citrate buffer (pH 3−4), succinate–NaOH

buffer (pH 4−6), phosphate buffer (pH 6−7.5), Tris–HCl buffer (pH 7.5−9) and glycine–NaOH buffer (pH 9−12) (Prim *et al*., 2000). The assay temperature was the optimum temperature of the enzyme. Optimum pH assays were repeated when the optimum temperature of the enzyme at the optimum pH obtained changed.

5.6.3 Thermostability

For thermostability assays, enzyme solutions prepared in 50 mM phosphate buffer (pH 7) were incubated for 1 h−40 days at the following assay temperatures (ºC): 4, 20, 30, 37, 45, 50, 60, 70, 80 and 100 (Prim *et al*., 2000). After incubation, the enzyme solutions were assayed at the optimum temperature and pH of the enzyme by the previously described classical-endpoint fluorimetric liquid assay.

5.6.4 pH stability

For pH stability assays, enzyme solutions were prepared in the corresponding buffers (at 50 mM): citrate buffer (pH 3−4), succinate–NaOH buffer (pH 4−6), phosphate buffer (pH 6−7.5), Tris–HCl buffer (pH 7.5−9) and glycine–NaOH buffer (pH 9−12), and were incubated for 1 h at RT (Prim *et al*., 2000). Enzyme solutions were then assayed at the optimum temperature and pH of the enzyme by the previously described classical-endpoint fluorimetric liquid assay.

5.6.5 Substrate range

The substrate range of the enzymes was determined by the previously described colorimetric assay, or by new colorimetric microassay. Both assays were performed at the optimum temperature of the enzyme and at pH 7. The substrates used were *p*-NP-derivatives with a carbon chain length of 2−18: *p*-NP acetate (C_2), *p*-NP butyrate (C_4), *p*-NP valerate (C_5), *p*-NP caproate (C_6), *p*-NP caprylate (C_8), *p*-NP caprate (C_{10}), *p*-NP laurate (C_{12}), *p*-NP palmitate (C_{16}) and *p*-NP stearate (C_{18}).

When the colorimetric assay was used, ($C_{2–12}$) *p*-NP-derivatives were assayed at a final concentration of $2.7 \cdot 10^{-2}$% (w/v or v/v), whereas ($C_{12–18}$) *p*-NP-derivatives were assayed at a final concentration of $1.35 \cdot 10^{-2}$% (w/v or v/v). Activity on *p*-NP laurate (C_{12}) (determined at both concentrations) was used to correlate the activity of *p*-NP-derivatives assayed at different concentrations (Prim *et al*., 2000).

When the substrate range was assayed by the new colorimetric microassay, all *p*-NP-derivatives were assayed at 1 mM (final concentration).

5.6.5 Enzyme kinetics

Enzyme kinetic parameters (V_{max}^{app}, K_M^{app}, etc) were calculated from the activity *vs*. substrate concentration curves by regression analysis performed using the software Sigma-Plot 8.0 (SPSS). Activity curves were obtained by the classical-endpoint fluorimetric assay, by the new colorimetric microassay, or by the HPLC assay, all of them performed at the optimum temperature and pH of the enzyme.

5.7 ACTIVATION/INHIBITION OF LIPASE ACTIVITY

Lipase inhibition or activation experiments were performed essentially as described (Grippa *et al*., 1999; Prim *et al*., 2000), using the previously described colorimetric assay, new colorimetric microassay, or HPLC assay.

Compounds under assay where solved in a proper solvent (see Table M.14) such as water, isopropanol, methanol, acetone, or dimethyl sulfoxide, and then added to reaction mixtures without enzyme, at the corresponding concentrations. After a preincubation of the resulting reaction mixtures, the enzyme solutions (also preincubated) were added to perform the enzymatic reactions as previously described. Exceptionally, inhibition by amino acid modifying agents such as PMSF (phenylmethylsulfonyl fluoride; serine), NAI (*N*-acetylimidazole; tyrosine), NBS (*N*-

bromosuccinimide; tryptophan) and PHMB (*p*-hydroxymercuribenzoic acid; cysteine) was performed by preincubating a reaction mixture with the proper inhibitor concentration and with enzyme, but without substrate, for 15 min at RT (Prim *et al*., 2000). Then, the substrate was added to perform the reaction as previously described.

Table M.14 Compounds which effect was assayed on lipolytic activity.

Cations (distilled water)

- Ag^{+} ($AgNO_3$)
- Ba^{2+} ($BaCl_2$)
- Ca^{2+} ($CaCl_2$)
- Co^{2+} ($CoCl_2$)
- Cu^{2+} ($CuSO_4$)
- Fe^{2+} ($FeCl_2$)
- Hg^{2+} ($HgCl_2$)
- Mg^{2+} ($MgCl_2$)
- Mn^{2+} ($MnCl_2$)
- Na^{+} (NaCl)
- NH_4^{+} (NH_4Cl)
- Ni^{2+} ($NiCl_2$)
- Pb^{2+} ($Pb(CH_3COO)_2$)
- Zn^{2+} ($ZnCl_2$)

Amino acid modifying agents

- NAI (distilled water)
- NBS (distilled water)
- PHMB (distilled water)
- PMSF (isopropanol)

Other agents (distilled water□

- EDTA
- Phytic acid
- SDS
- Triton X-100
- Urea

Natural substances

♦ **Saturated fatty acids** (isopropanol)

- Capric acid (C_{10})
- Lauric acid (C_{12})
- Myristic acid (C_{14})

♦ **Saponins**

- β-Aescin (methanol)
- Digitonin (methanol)
- Glycyrrhizic acid (GA) (dimethyl sulfoxide)
- *Quillaja* saponin (□S) (distilled water)

♦ **Flavonoids**

- 3-hydroxyflavone (3-HF) (acetone)
- 5-hydroxyflavone (5-HF) (acetone)
- (±)-Catechin (methanol)
- Kaempferol (dimethyl sulfoxide)

♦ **Alkaloids**

- Aspidospermine (methanol)
- Papaverine (distilled water)
- Physostigmine (methanol)
- Pilocarpine (distilled water)
- Raubasine (methanol)
- Rescinnamine (methanol)
- Reserpine (methanol)
- Trigonelline (distilled water)

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 solvent to take into consideration the effect of each solvent on 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.