APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Sept. 1997, p. 3553–3560 Vol. 63, No. 9
0099-2240/97/$04.00+0

Genetic and Biochemical Characterization of a New Extracellular Lipase from *Streptomyces cinnamomeus*

PATRICIA SOMMER, CHRISTIANE BORMANN, AND FRIEDRICH GÖTZ*

Mikrobielle Genetik, Universität Tübingen, D-72076 Tübingen, Germany

Received 31 March 1997/Accepted 1 July 1997

***Streptomyces cinnamomeus* Tü89 secretes a 30-kDa esterase and a 50-kDa lipase. The lipase-encoding gene, *lipA*, was cloned from genomic DNA into *Streptomyces lividans* TK23 with plasmid vector pIJ702. Two lipase-positive clones were identified; each recombinant plasmid had a 5.2-kb *Mbo*I insert that contained the complete *lipA* gene. The two plasmids differed in the orientation of the insert and the degree of lipolytic activity produced. The *lipA* gene was sequenced; *lipA* encodes a proprotein of 275 amino acids (29,213 Da) with a pI of 5.35. The LipA signal peptide is 30 amino acids long, and the mature lipase sequence is 245 amino acids long (26.2 kDa) and contains six cysteine residues. The conserved catalytic serine residue of LipA is in position 125. Sequence similarity of the mature lipases (29% identity, 60% similarity) was observed mainly in the N-terminal 104 amino acids with the group II *Pseudomonas* lipases; no similarity to the two *Streptomyces* lipase sequences was found. *lipA* was also expressed in *Escherichia coli* under the control of *lacZ* promoter. In the presence of the inducer isopropyl-β-D-thiogalactopyranoside (IPTG), growth of the *E. coli* clone was severely affected, and the cells lysed in liquid medium. Lipase activity in the *E. coli* clone was found mainly in the pellet fraction. In sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis, three additional protein bands of 50, 29, and 27 kDa were visible. The 27-kDa protein showed lipolytic activity and represents the mature lipase; the 29- and 50-kDa forms showed no activity and very probably represent the unprocessed form and a dimeric misfolded form, respectively. For higher expression of *lipA* in *S. lividans*, the gene was cloned next to the strong *aph*II promoter. In contrast to the *lipA*-expressing *E. coli* clone, *S. cinnamomeus* and the corresponding *S. lividans* clone secreted only an active protein of 50 kDa. The lipase showed highest activity with C_6 and C_{18} triglycerides; no activity was observed with phospholipids, Tween 20, or *p*-nitrophenylesters. Upstream of *lipA* and in the same orientation, an open reading frame, *orfA*, is found whose deduced protein sequence (519 amino acids) shows similarity to various membrane-localized transporters. Downstream of *lipA* and in the opposite orientation, the open reading frame *orfB* (encoding a 199-amino-acid protein) is found, which shows no conspicuous sequence similarity to known proteins, other than an NAD and flavin adenine dinucleotide binding-site sequence.**

Bacterial lipases (triacylglycerol acylhydrolase; EC 3.1.1.3) catalyze the hydrolysis of triglycerides into diglycerides, monoglycerides, glycerol, and fatty acids, and under certain conditions they also catalyze the reverse reaction, esterification, forming glycerides from glycerol and fatty acids. Some lipases are also able to catalyze transesterification and enantioselective hydrolysis reactions.

Interest in lipases has increased in the past decades, especially with regard to the possible applications of lipases in industry. The number of bacterial lipases that have been purified and characterized and whose genes have been sequenced is increasing (for reviews, see references 10 and 27). For gram-positive bacteria, the (phospho)lipases of various *Staphylococcus*, *Bacillus*, and *Streptomyces* species have been studied. The staphylococcal lipases are organized as preproenzymes with a molecular mass of 70 to 77 kDa. The lipase propeptide is, at least in *Staphylococcus hyicus*, extracellularly processed by a specific protease to the 46-kDa mature form, which contains the proposed catalytic triad Ser-His-Asp (2, 28). The propeptide region is essential for efficient secretion and proteolytic stabilization of the lipase (15, 37). The *S. hyicus* and *Staphylococcus aureus* lipases are Ca^{2+} dependent; they differ, however, in their substrate specificity (39, 50, 55). The *S. hyicus* lipase prefers phospholipids as the substrates and hydrolyzes neutral lipids irrespective of their chain length. In contrast, the *S. aureus* lipase has a narrow substrate specificity; short-chain triacylglycerols and acyl esters of both *p*-nitrophenol and umbelliferone are readily degraded, whereas medium- and long-chain lipids and phospholipids are poor substrates.

Bacillus subtilis 168 secretes a very small lipase with a molecular mass of only 19.4 kDa (14). The enzyme has a preference for the 1,3 position and prefers fatty acids with a chain length of eight carbons. There is a great biotechnological interest in this enzyme because of its remarkable alkaline stability (pH 12) and activity (36). Another species, *Bacillus thuringiensis*, produces a 38-kDa phosphatidylinositol-specific phospholipase C (33, 35). Two *Streptomyces* lipase sequences have been published, the 28-kDa lipase of *Streptomyces* sp. strain M11 (42) and the similar-sized lipase of *S. albus* (11); the lipases exhibit 82% sequence identity and contain two cysteine residues. Little is known about their substrate specificity.

While lipases (triacylglycerol acyl hydrolases) act preferentially on emulsified substrates with long-chain fatty acids, the carboxylesterases (EC 3.1.1.1) hydrolyze water-soluble or emulsified esters with relatively short fatty acid chains. These hydrolases all contain a similar catalytic triad, generally consisting of a nucleophilic serine residue that acts in conjunction with a histidine residue and an aspartic acid residue (8, 13).

Two *Streptomyces* esterases have been analyzed and cloned:

* Corresponding author. Mailing address: Mikrobielle Genetik, Universität Tübingen, Waldhäuser Str. 70/8, D-72076 Tübingen, Germany. Phone: (49)-7071-2974636. Fax: (49)-7071-295937. E-mail: friedrich.goetz@uni-tuebingen.de.

the 345-amino-acid esterase from *S. scabies* and the 326-amino-acid esterase from *S. diastatochromogenes* (44, 53). The *S. diastatochromogenes* esterase hydrolyses *p*-nitrophenylcaprylate and has optimum activity at pH 9.0 and 40 to 50°C. The activity of the esterase is almost completely inhibited by phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate but is unaffected by iodoacetamide, ethylmaleimide, and EDTA. Interestingly, the two *Streptomyces* esterases lack the conserved motif GXSXG, which carries the active-site serine of hydrolytic enzymes. A serine modified by [1,3-^{3}H]diisopropyl fluorophosphate has been identified by sequencing the amino-terminal region of the *S. diastatochromogenes* esterase. The serine is located at position 11 of the mature enzyme in the sequence GDSYT. This finding and results obtained by site-directed mutagenesis studies suggest that S-11 might be the active-site nucleophile. Streptomycetes also produce lipase inhibitors. Most of the inhibitors are active only against some eukaryotic lipases. Lipstatin, produced by *S. toxytricini*, irreversibly inhibits pancreatic lipase (22, 58). The molecule has a β-lactone structure, which is necessary for inhibition, incorporated into a hydrocarbon backbone. An esterase inhibitor, valilactone, produced by a strain closely related to *Streptomyces albolongus*, also has activity against hog pancreatic lipase (31). Tetrahydrolipstatin, a reduced form of lipstatin, inhibits a wider range of eukaryotic lipases but has no effect on the extracellular lipases from *Staphylococcus aureus* and *Rhizopus arrhizus* (6). Until now, only two very homologous streptomycete lipases have been genetically characterized.

Here we describe the gene locus, the sequence, the activity as a dimer and as a monomer, and the substrate specificity of a new lipase from *Streptomyces cinnamomeus*. The results suggest that the variability of this class of enzymes is much higher than expected in this bacterial group.

MATERIALS AND METHODS

Strains and plasmids. *S. cinnamomeus* Tü89 was obtained from H. Zähner (University of Tübingen, Tübingen, Germany). *S. lividans* TK23 was obtained from D. A. Hopwood (John Innes Institute, Norwich, England) and used as a host for constructing gene libraries of *S. cinnamomeus* in plasmid pIJ702 (30). The vectors used for subcloning were pUC19 (60) and pWHM3, a *Streptomyces*/*Escherichia coli* shuttle vector (56) obtained from C. R. Hutchinson (University of Wisconsin, Madison, Wis.). Plasmid pJOE865 (3), which contains the 1.3-kb *Hin*dIII-*Sma*I fragment with the *aph* gene from Tn*5*, was kindly provided by J. Altenbuchner (University of Stuttgart, Stuttgart Germany).

Media and culture conditions. Strains of *E. coli* were grown in Luria-Bertani (LB) medium (5 g of NaCl, 5 g of yeast extract [Gibco], and 10 g of Bacto-Tryptone [Difco] per liter) or 2xYT (16 g of Bacto-Tryptone [Difco], 10 g of yeast-extract [Gibco], and 5 g of NaCl per liter). For plates, LB medium was solidified with 1.8% (wt/vol) agar. *S. lividans* was cultivated on HA plates, which consisted of 0.4% glucose, 1% malt extract, 0.4% yeast extract, and 1.8% agar (pH 7.2). For preparation of protoplasts and DNA isolation, *Streptomyces* strains were grown in CRM (10.3% sucrose, 2% tryptic soy broth, 1% yeast extract [pH 6.6] [43]) liquid medium. For protoplast regeneration, R3 agar plates were used (49). Thiostrepton was added to solid media at 30 mg/liter and to liquid media at 10 mg/liter for selective pressure when recombinant strains were grown. Thiostrepton was a gift from S. J. Lucania (Squibb, Princeton N.J.). *Streptomyces* strains were grown at 27°C *E. coli* strains were grown at 37°C in LB liquid medium and on LB agar plates (46), both supplemented with ampicillin (100 mg/liter).

Chemicals. Restriction enzymes were obtained from various commercial sources, and T4 DNA ligase was obtained from Gibco-BRL. All were used as recommended by the manufacturer. Lipase substrates were obtained from Sigma (Deisenhofen, Germany); [α-^{35}S]dATP was purchased from ICN Biomedicals Inc.

Lipase detection and characterization. Three types of lipase indicator plates were used. (i) For tricaprylin plates, tricaprylin (1%, vol/vol) was added to 2xYT agar. Lipase production on these plates was indicated by a zone of clearance around the colonies. (ii) For Tween 20 plates (for esterase activity), Tween 20 (1%, vol/vol) was added to tributyrin agar. Esterase production on these plates was indicated by a powder-like zone around the colonies. (iii) For olive oil plates, olive oil (1%, vol/vol) and rhodamine B (1 μg) were added to 2xYT agar. Production of lipase on these plates was detectable only after at least 4 days of incubation and is detected by the formation of a orange-colored fluorescent halo (under UV light at 350 nm) around the colonies (adapted from reference 32). For determination of the substrate specificity, the following compounds were used: tributyrin ($C_{4:0}$), tricaproin ($C_{6:0}$), tricaprylin ($C_{8:0}$), tricaprin ($C_{10:0}$), trilaurin ($C_{12:0}$), trimistyrin ($C_{14:0}$), tripalmitin ($C_{16:0}$), and triolein [$C_{18:1\ (cis)\text{-}9}$]. The substrates were emulsified with Branson 250 Sonifier for 3 min (duty cycle 70%). *p*-Nitrophenylcaprylate, *p*-nitrophenyllaurate, and *p*-nitrophenylpalmitate (50 mM Tris-HCl [pH 9], 0.1% Triton X-100, 5 mM *p*-nitrophenyl) were tested.

General DNA techniques. Recombinant DNA techniques in *Streptomyces* spp. were performed as described by Hopwood et al. (24). Total DNA from *Streptomyces* was isolated by the large-scale method reported by Hunter (25). Standard procedures were used for *E. coli* (46). *E. coli* cells were transformed (17) with the Gene Pulser Apparatus (Bio-Rad, Munich, Germany). Plasmid DNA was isolated on Nucleobond AX100 columns (Macherey-Nagel, Düren, Germany). DNA fragments were isolated from agarose gels with GeneClean (Bio 101). To clone a lipase-encoding gene from *S. cinnamomeus*, total DNA was partially digested with *Mbo*I, and 5- to 10-kb fragments were ligated to the multicopy plasmid pIJ702, which was cleaved at the unique *Bgl*II site within the *mel* gene. The ligation mixture was used to transform *S. lividans* TK23. Thiostrepton-resistant Mel$^-$ transformants were grown for 48 h on tricaprylin plates. Colonies that hydrolyzed tricaprylin produced halos around the colonies.

The nucleotide sequence was determined by the dideoxy chain termination method (47) with the T7 sequencing kit, deaza-dGTP reaction mixtures (Pharmacia), and [^{35}S]dATP or with the AutoRead Sequencing kit (Pharmacia) for automated sequencing with the A.L.F. Sequencer (Pharmacia). A combination of subclones constructed in pUC19 (60) with the M13/pUC universal and reverse primer and synthetic oligonucleotide primers were used. The DNA and deduced amino acid sequence were analyzed with the sequence analysis software package of the Genetics Computer Group, University of Wisconsin, Madison (16), and MacDNAsis Pro (Hitachi Software Engineering, San Bruno Calif.). The similarity of the deduced amino acid sequence from *lipA* to those of proteins in databases (PDP, SwissProt, SPupdate, PIR, GenPept, GPupdate, release 145) was determined with the FASTA (41) and BLASTP (1) programs.

General protein techniques. Proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 12% polyacrylamide resolving gel and a 4% polyacrylamide stacking gel (34). Molecular mass markers were obtained from Bio-Rad and Sigma: carbonic anhydrase (29 kDa), ovalbumin (45 kDa), bovine serum albumin (66 kDa), phosphorylase *b* (97.4 kDa), β-galactosidase (116 kDa), and myosin (205 kDa). Proteins were visualized in the gels by staining with Coomassie brilliant blue. When activity staining was performed, the sample applied to the gel was not boiled, and after electrophoresis, SDS was removed by washing the gel once for 20 min with 20% isopropanol and twice for 10 min with distilled water.

For lipase activity detection, the gel was transferred to a 1.3% agar plate containing 1% tributyrin or tricaprylin, 25 mM Tris-HCl (pH 8.0), and 5 mM $CaCl_2$; for esterase activity, 1% Tween 20–25 mM Tris-HCl (pH 8.0) was used as a substrate. After incubation for about 1 to 3 h at 37°C, lipase activity was visualized by clearing bands of tributyrin or tricaprylin hydrolysis, and esterase activity was seen as bands of precipitated fatty acids.

To assay the extracellular lipase and esterase activity of the *Streptomyces* clones, the proteins of 20-ml culture supernatants were concentrated by acetone precipitation at 4°C. The precipitate was dissolved in 1.0 ml of 10 mM Tris-HCl (pH 8.0).

With *E. coli*(pUPS7), the proteins (also of inclusion bodies) were solubilized essentially as described in the pET system manual (Novagen). The cells were cultivated overnight in 50 ml of LB medium (without isopropyl-β-D-thiogalactopyranoside [IPTG]) centrifuged, and the pellet was resuspended in 1/10 volume of 50 mM Tris-HCl (pH 8)–0.2 mM EDTA. After addition of lysozyme (100 mg/ml) and 1/10 volume of 1% Triton X-100, the cell suspension was incubated at 30°C for 15 min. Subsequently, the cell suspension was cooled to 4°C and centrifuged at 12,000 × *g* for 15 min. The supernatant contained the soluble proteins, and the pellet contained the insoluble cell fractions. Lipase activity was found essentially in the pellet fraction. The soluble fraction was concentrated approximately 50-fold before being subjected to SDS-PAGE, and the pellet was resuspended in 1 ml of sample buffer.

Protein concentrations were measured by the method of Bradford (7) with bovine serum albumin as a standard. For N-terminal sequencing of proteins separated by SDS-PAGE, proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon P; Millipore) and sequenced in a model 477A pulsed-liquid protein sequencer (Applied Biosystems) equipped with a model 120A on-line phenylthiohydantoin amino acid analyzer.

Nucleotide sequence accession number. The nucleotide sequence of the 3,768-bp sequence of *S. cinnamomeus* Tü89 encoding *lipA*, *orfA*, *orfB*, and the truncated *orfX* is U80063.

RESULTS

Cloning and sequencing of the *S. cinnamomeus* lipase gene (*lipA*). DNA from *S. cinnamomeus* Tü89 was partially cleaved with *Mbo*I, and the resulting DNA fragments (5 to 10 kb) were inserted into the *Bgl*II site (located within the *mel* gene) of

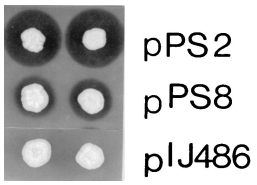

FIG. 1. Lipase activity on tricaprylin plates. *S. lividans*(pPS2), *S. lividans* (pPS8), and, as a negative control, *S. lividans*(pIJ486) were cultivated for 72 h. Lipolytic activity is indicated by a halo around the colony.

pIJ702 and transformed into *S. lividans* TK23. Transformants were selected on thiostrepton-containing agar plates; DNA insertion was indicated by white colonies. The colonies were transferred to olive oil-rhodamine B plates. Of 3,000 clones tested, 2 (containing plasmid pPS2 or pPS8) showed weak lipolytic activity, indicated by the formation of a fluorescent halo around the colonies. Both lipase-positive clones lost lipase activity with a high frequency when cultivated on 2xYT agar plates in the presence of olive oil-rhodamine B; after overnight cultivation, the plasmids suffered deletions in 20% of more than 100 clones analyzed. The deletions were of different sizes, but the lipase-negative phenotype suggests that the *lipA* gene was always affected. However, no instability was observed when the clones were cultivated on tributyrin or tricaprylin plates or in medium with no lipase substrate. On tricaprylin plates, the two clones differed in the degree of lipolysis: the pPS8-containing *S. lividans* clone was less active than the clone with pPS2 (Fig. 1).

Both plasmids contained a 5.2-kb insert in different orientation (Fig. 2A). A 2.6-kb (*Pvu*II-*Kpn*I) fragment which was common to both plasmids and which most probably contained *lipA* was subcloned in *E. coli* by using the *Sma*I site of pUC18, forming pUPS6 and pUPS5; in plasmid pUPS6, *lipA* was in-frame with *lacZ*, and in pUPS5, *lipA* was in the opposite direction. *E. coli* containing either plasmid showed no lipolytic activity, very probably because the *lipA* promoter is not recognized, and *lipA* is flanked by a putative transcription termination sequences.

The DNA insert was sequenced; the *lipA*-containing DNA and protein sequences are shown in Fig. 3. The reading frame of *lipA* contains 825 bp (70.3 mol% G+C), which encodes a protein of 275 amino acids with a theoretical mass of 29,213 Da and a pI of 5.35. There is a putative signal sequence of 30 amino acids and a characteristic signal peptidase-processing site, Ala-X-Ala[30]. For the 245-amino-acid processed lipase, a molecular mass of 26.2 kDa is predicted.

A comparison of the LipA sequence with known lipases revealed high similarity around the proposed catalytic serine-G-(H)-S[125]-(Q)-G-, which is highly conserved among lipases and esterases (9, 10, 28). A good sequence similarity was observed with the group II *Pseudomonas* lipases, which are in a similar size range of 280 to 290 amino acids (27). A comparison of the LipA sequence with lipases from three different *Pseudomonas* species revealed an identity of 29% and a similarity of 60% within the first 104 N-terminal amino acids, which contain the active-site serine (Fig. 4). Cloned *Streptomyces* lipase genes of other origins showed no or only little overall similarity.

A putative ribosome binding site with good complementarity to the 3′ end of the 16S rRNA of *S. lividans* (51) is found 7 bp upstream from the *lipA* start codon. A sequence that matches the -35 consensus sequence of *Streptomyces* promoters (TTG ACG) is found 116 bp upstream of the start codon; no sequences with similarity to the -10 promoter region were found (51).

Expression of *lipA* in *E. coli*. As mentioned above, the presence of the plasmids pUPS5 and pUPS6 in *E. coli* did not lead to a lipolytic activity with or without induction by IPTG. This

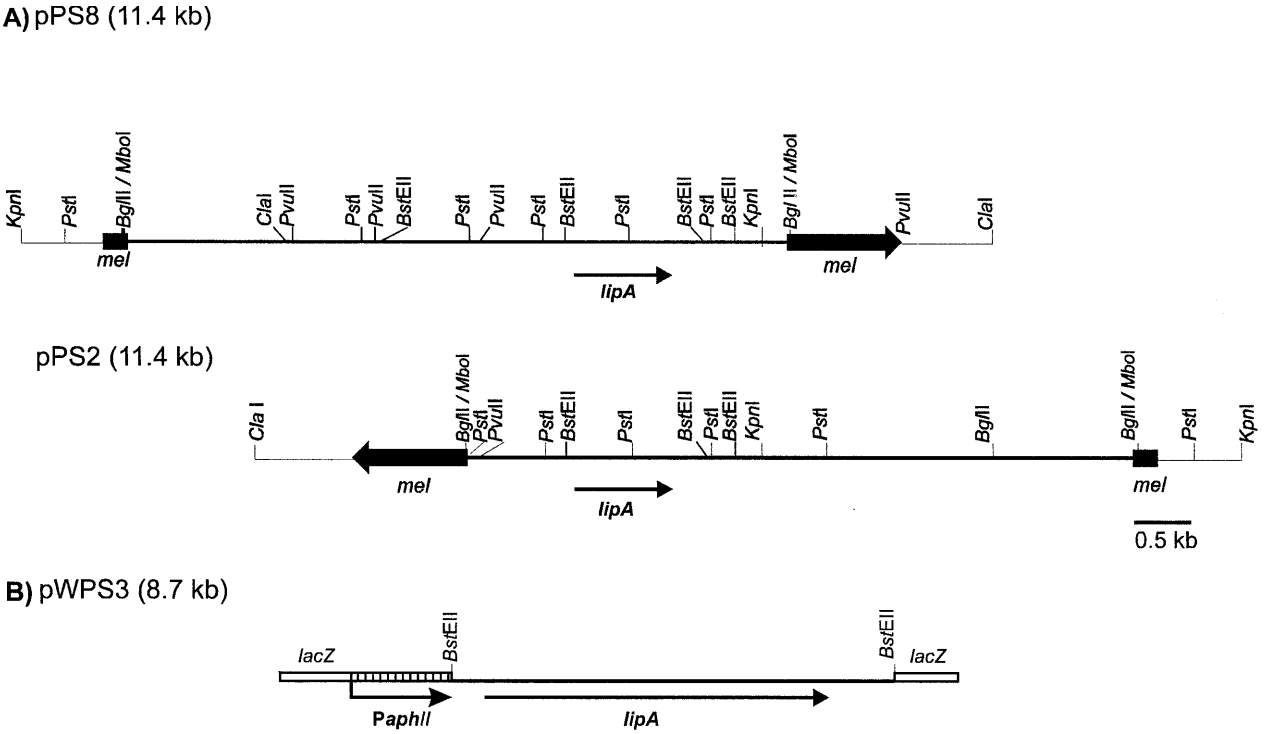

FIG. 2. Genetic organization of *lipA* in plasmids pPS8 and pPS2 (A) and pWPS3 (B). In pWPS3, *lipA* is under the transcriptional control of the *aph*II promoter.

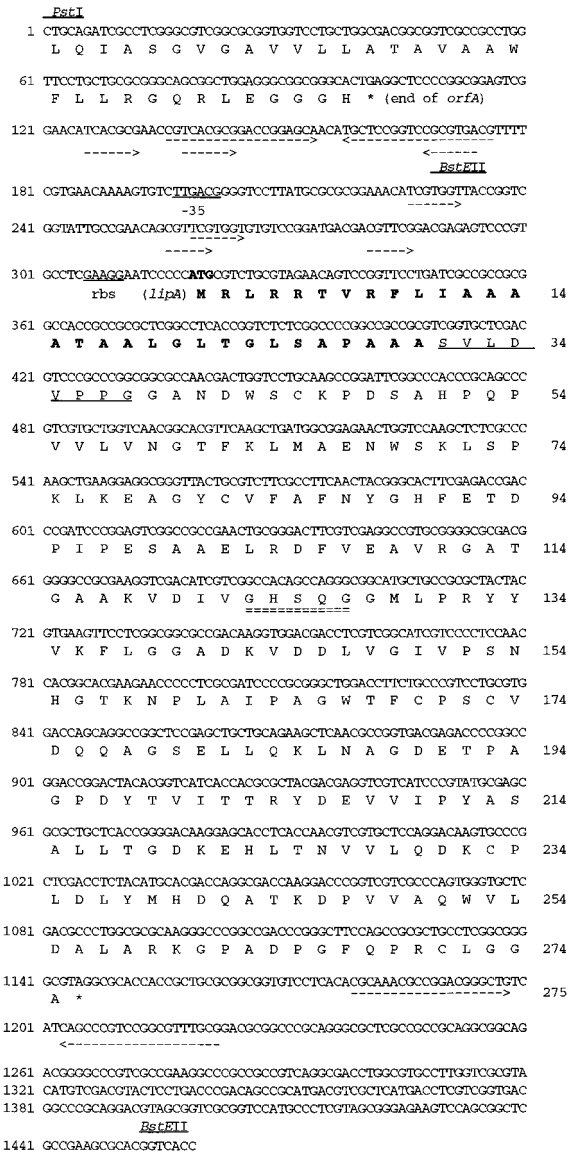

FIG. 3. Nucleotide sequence of the 1,459-bp *Pst*I-*Bst*EII fragment and amino acid sequence of LipA. The potential -35 promoter region and the ribosomal binding site (rbs) of *LipA* are indicated. The first amino acids of the mature LipA are underlined (single line), and the conserved active-site serine region is underlined (double line). Direct and inverted repeats are indicated by arrows. Upstream of *lipA* is the end of *orfA*. Both *orfA* and *lipA* have transcription terminator-like structures downstream of the gene.

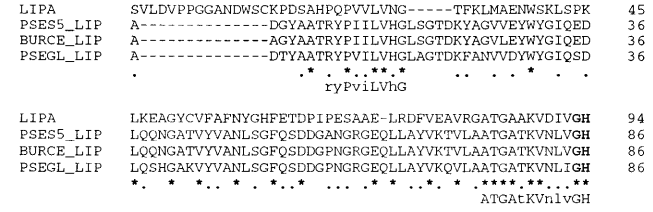

FIG. 4. Alignment of the first 104 amino acids of the mature *S. cinnamoneus* LipA and three *Pseudomonas* lipases. LIPA, lipase from *S. cinnamoneus* Tü89 (this work); PSES5_LIP, lipase from *Pseudomonas* sp. (26); BURCE_LIP, lipase from *Burkholderia cepacia* (*Pseudomonas cepacia*) (29); PSEGL_LIP, lipase from *Pseudomonas glumae* (20). The conserved amino acids around the active serine site are in boldface type. Identical amino acids are indicated by an asterisk, and similar amino acids are indicated by a dot.

is not surprising, since *lipA* in both orientations is flanked by a transcription terminator-like structure (Fig. 5). We therefore inserted the *lipA*-containing *Bst*EII fragment into the *Sma*I site of pUC19, thereby forming pUPS7 and pUPS8. In plasmid pUPS7, *lipA* was in-frame with *lacZ*, and in pUPS8, *lipA* was inserted in the opposite direction. Only *E. coli*(pUPS7) showed lipolytic activity on tricaprylin agar (not shown). Lipolytic activity was observed without induction; however, in the presence of IPTG, cell growth was severely affected, and the cells lysed in liquid culture. For the isolation of LipA, *E. coli*(pUPS7) was cultivated in LB medium without inducer. The proteins were solubilized essentially as specified for the pET-System, where the supernatant contained the soluble proteins and the pellet contained the insoluble cell fractions. Lipase activity was found essentially in the pellet fraction, which suggested that the lipase is associated with the cell wall fraction or that inclusion bodies were formed.

The samples were treated with sample buffer, boiled, and subjected to SDS-PAGE. While the soluble fraction revealed no additional protein bands compared to the control, the pellet fraction revealed three additional major protein bands of 50, 29, and 27 kDa (Fig. 6). Some of the proteins (also in the control) did not enter the gel; they may represent membrane-associated and/or aggregated proteins. Since a molecular mass of 26.2 kDa was calculated for the mature lipase, we expected that the 27-kDa form was the mature lipase. We isolated the 50-, 29-, and 27-kDa proteins by the polyvinylidene difluoride membrane technique and tried to obtain N-terminal sequences. No sequence was obtained with the 50- and 29-kDa proteins. The 27-kDa protein revealed an N-terminal sequence of SVLDVPPG, which corresponded to amino acid positions 31 to 39 of LipA (underlined sequence in Fig. 3). This indicates that the sequence AAA^{30} represents the signal peptidase processing site. The 29-kDa protein very probably represents the unprocessed form, and the 50-kDa protein form very probably represents a dimeric aggregate.

Expression of *lipA* in *S. lividans*. To express *lipA* in *S. lividans*, we inserted the *lipA*-containing *Bst*EII fragment in the *Eco*RI (made blunt ended) site of the shuttle plasmid pWHM3. We then inserted the *Hin*dIII-*Bgl*II fragment of pJOE865, which contains the *aph*II promoter of the aminoglycoside phosphotransferase gene of transposon Tn*5*, into the *Hin*dIII-

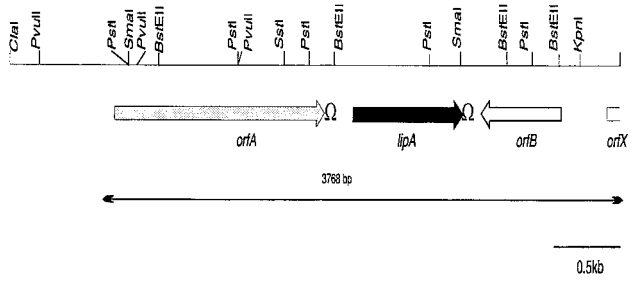

FIG. 5. Genetic organization of the regions flanking *lipA*. Upstream is the 1,556-bp *orfA*, which shows sequence similarity to certain membrane-localized transporters; downstream is the 795-bp *orfB*, whose function is unknown, and the truncated *orfX*. Ω, transcription terminator-like structure.

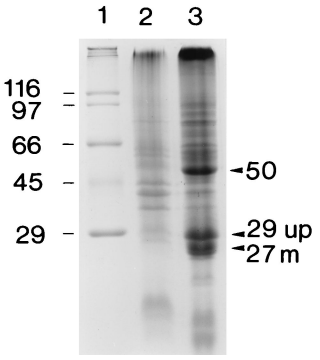

FIG. 6. Coomassie blue-stained SDS-polyacrylamide gel after electrophoresis of the pellet (insoluble) fraction of an *E. coli* clone. Lanes: 1, marker proteins; 2, negative control *E. coli*(pUC19); 3, *E. coli*(pUPS7). 27 m, 29 up, and 50 most probably represent the 27-kDa mature lipase, the 29-kDa unprocessed lipase, and the 50-kDa dimer, respectively. Only the 27-kDa form shows lipolytic activity.

*Bam*HI sites. In the resulting plasmid, pWPS3, *lipA* is under transcriptional control of the strong *aph*II promoter (Fig. 2B).

To investigate the lipolytic activity, *S. cinnamomeus* Tü89, *S. lividans*(pWHM3), and *S. lividans*(pWPS3) were cultivated in CRM liquid medium for 72 h. The proteins in the culture supernatant were concentrated by acetone precipitation and subjected, together with the insoluble protein fraction of *E. coli* (pUPS7) and *E. coli*(pUC18), to SDS-PAGE. The SDS-polyacrylamide gel was washed, laid onto $CaCl_2$-containing tributyrin agar, and incubated for 12 h at 37°C. As shown in Fig. 7, the culture supernatants of *S. cinnamomeus* Tü89 and *S. lividans*(pWPS3) revealed a band with lipolytic activity at 50 kDa, which is in contrast to the *E. coli*(pUPS7) pellet extract, where the 27-kDa protein represents the active form. So far, we have been unsuccessful in determining the N-terminal amino acid sequence of the extracellular active 50-kDa protein of *S. cinnamomeus* Tü89; this form probably represents a dimer. We do not know whether the dimer is held together by disulfide bridges (LipA contains six cysteine residues) or other forces. We only have observed that after boiling in sample buffer (under reducing conditions), the 50-kDa form of *S. lividans* (pWPS3) is still present. The inactive 50-kDa form in *E. coli* might be generated by misfolding in inclusion bodies.

In Fig. 7 (lane 7), the separated proteins of *S. cinnamomeus* Tü89 were also tested on Tween 20 (a substrate for esterases)-containing agar plates. An activity band of 30 kDa was visible, which indicated that this strain produced not only the lipase but also an esterase. Lipases and esterases frequently contain one or two cysteine residues near other cysteine residues that are involved in disulfide bridging (13). Recently, the crystal structure of the *S. scabies* esterase was determined and revealed that the esterase is stabilized by three disulfide bridges formed between the six cysteines of the protein by linking two subsequent residues (57).

Substrate specificity of LipA. We investigated the hydrolytic activity of *S. cinnamomeus* Tü89, *S. lividans*(pWHM3), and *S. lividans*(pWPS3) by using various substrates. With the chromogenic substrates *p*-nitrophenylcaprylate, *p*-nitrophenyllaurate, and *p*-nitrophenylpalmitate, activity was observed only with *S. cinnamomeus* Tü89; this indicated that this hydrolytic activity very probably comes from the esterase, which also shows activity with Tween 20.

In the plate assay, we tested the following compounds: the triglycerides tributyrin ($C_{4:0}$), tricaproin ($C_{6:0}$), tricaprylin ($C_{8:0}$), tricaprin ($C_{10:0}$), trilaurin ($C_{12:0}$), trimistyrin ($C_{14:0}$), tripalmitin ($C_{16:0}$), and triolein [$C_{18:1\ (cis)-9}$] and the monolaurate Tween 20 (Fig. 8). *S. cinnamomeus* Tü89 showed good activity with all substrates; the highest activities were obtained with C_4, C_6, C_8, C_{18}, and Tween 20 as substrates. *S. lividans*(pWHM3) showed only a weak activity with tributyrin. *S. lividans*(pWPS3) revealed the highest activity with C_6 and C_{18}, followed by C_8 and C_4. With the C_{10} and C_{12} triglycerides, only small halos were formed. The lowest activity was observed with C_{14} and C_{16} as substrates (data not shown); with Tween 20, no activity was detectable. The *lipA* clones also showed no activity with phospholipids in the agar plate assay with lecithin as the substrate or in the colorimetric assay with 2-hexadecanoylthiopropane 1-phosphocholine (50) as the substrate.

The broader substrate specificity of *S. cinnamomeus* Tü89 is very probably due to the lipase and esterase activity. We cannot rule out the possibility that there are more hydrolytic enzymes involved. On the other hand, only one activity band (50 kDa) with tributyrin and one (30 kDa) with Tween 20 as a substrate were identified in the SDS-polyacrylamide gel.

***lipA*-flanking DNA region. (i) *orfA*.** The DNA region flanking *lipA* was also sequenced. The genetic map of the 4.6-kb fragment is shown in Fig. 5. Upstream of *lipA*, in the same orientation and separated by a transcription terminator-like sequence, is *orfA* (1,556 bp). The derived protein (519 amino acids) shows sequence similarity to various membrane-localized transporters involved in the transport of antibiotics, sugars, and other substances (21). Among the compared transporters with sequence similarity are the methyl viologen resistance protein of *Salmonella typhimurium* (accession no. P37549 [23]), the transport protein for intercalating substances from *Staphylococcus aureus* (P23215 [45]), the multidrug resistance protein from *Streptomyces pristinaespiralis* (X84072 [5]), the actinorhodin-resistance protein from *Streptomyces coelicolor* (S18539 [18]), and the proton antiporter efflux pump (LfrA) of *Mycobacterium smegmatis* (U40487: [52]). The identity of OrfA to these transporters was 38% (mean value). The highest identity (45%) was observed with the LfrA of *Mycobacterium smegmatis*. Furthermore, with the program SOAP (PC/GENE), it was predicted that OrfA contains 12 transmembrane helices.

(ii) *orfB*. Downstream of *lipA*, in the opposite orientation and separated by a transcription terminator-like sequence, is the 597-bp *orfB* (Fig. 5). The derived protein (199 amino acids, pI 10.02) shows no significant sequence similarity to proteins in the data bank. The only sequence similarity found was to a conserved nucleotide-binding domain (GXGXXG) in the N-terminal portion, which represents the first βαβ unit in the

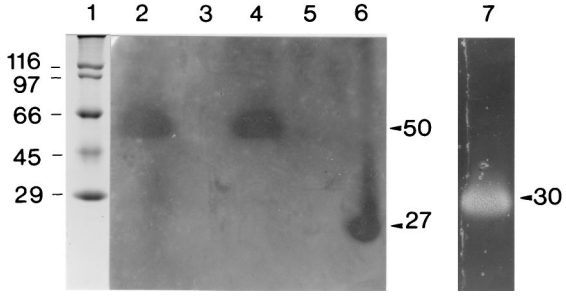

FIG. 7. Lipase activity staining after SDS-PAGE. Lanes: 1, marker proteins; 2, culture supernatant of *S. cinnamomeus* Tü89; 3, *S. lividans*(pWHM3); 4, *S. lividans*(pWPS3); 5, *E. coli*(pUC19); 6, *E. coli*(pUPS7); 7, *S. cinnamomeus* Tü89. In lanes 1 to 6, tributyrin was used as a substrate; in lane 7, Tween 20 was used as a substrate. The gels were incubated for 12 h. The protein samples of the *E. coli* clones were from the pellet fraction, and those of the *Streptomyces* strains were from the culture supernatant.

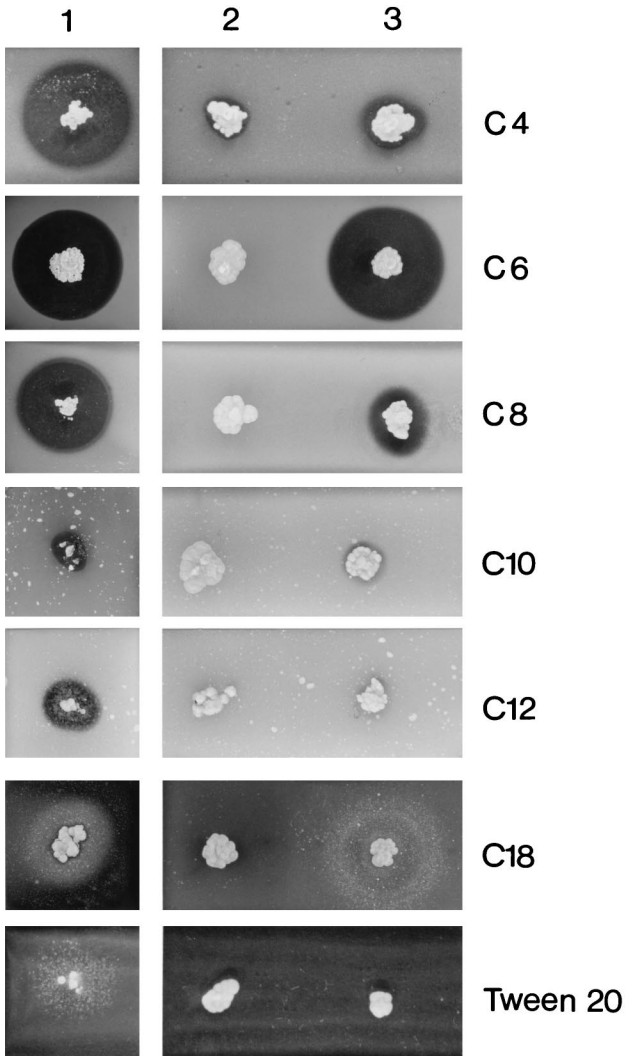

FIG. 8. Lipolytic activity with various substrates of *S. cinnamomeus* Tü89 (column 1), *S. lividans*(pWHM3) (column 2), and *S. lividans*(pWPS3) (column 3). C4 to C18 indicate the triglycerides with the corresponding acyl chain length. The agar plates (2xYT) contained 1% substrate and were incubated for 2 days.

Rossman folding of NAD and flavin adenine dinucleotide binding proteins (48).

Upstream of *orfB* and in the opposite orientation is the truncated *orfX*. It is notable that the 290-bp intervening sequence between *orfB* and *orfX* is distinguished by a relatively low G+C content (61%) and an accumulation of direct and indirect repeats.

DISCUSSION

A remarkable observation was that the *lipA*-containing plasmids pPS8 and pPS2 showed only an instability (loss of lipase activity) in the *S. lividans* clones when cultivated in the presence of olive oil-rhodamine B but not when cultivated with tributyrin or tributyrate or without a lipase substrate. Obviously, it is not the lipase activity per se or the products of the two triglycerides that act deleteriously; the instability very probably comes from the reaction with olive oil-rhodamine B. One explanation is that toxic mono- or diglycerides are formed from olive oil by lipase activity and that these toxic compounds exert a selective pressure on lipase-negative variants.

The *S. lividans* clone with pPS2 showed a much larger halo on tricaprylin plates than did the clone with pPS8. This observation was at first astonishing, since both plasmids contained *lipA* and the cloned fragments were nearly the same size. However, in pPS8, *lipA* is in frame with the plasmid-encoded *mel* gene, and an intact open reading frame (*orfA*) is located upstream of *lipA*. In pPS2, *orfA* is truncated and *lipA* is in an orientation opposite to that of *mel*. In principle, one would expect that because of transcriptional readthrough, *lipA* expression would be higher in the pPS8 clone. Since the opposite was found, we assumed that the gene product of *orfA* exerts a negative influence on *lipA* expression or lipase production, either directly or indirectly. The *lipA* promoter region contains a number of direct and indirect repeat sequences (Fig. 3), which may represent potential regulatory motifs. Similar motifs were found in the *galP1* promoter region, where they apparently act as an operator for negative regulation of transcription (38). As mentioned above, OrfA shows sequence similarity to various membrane-localized transporters involved in the transport of antibiotics, sugars, or protons. Twelve transmembrane helices are predicted based on the amino acid sequence. Proton-dependent transport proteins usually consist of one protein that contains 12 transmembrane helices (4, 21, 45). The proton-dependent transport proteins studied to date are distinguished by a conserved cytoplasmic loop, which is proposed to be involved in the proton-dependent change in conformation. Besides this conserved loop structure, there are also four conserved domains in the N-terminal portion of the proteins, and these domains are also present in OrfA. We therefore propose that *orfA* encodes a transmembrane, proton-dependent transport protein. Since *S. cinnamomeus* Tü89 produces the polyketid antibiotic kirrothricin (54), we speculated that *orfA* might be involved in kirrothricin export and probably in kirrothricin resistance. However, heterologous expression in *S. lividans* did not lead to kirrothricin resistance. Since there is frequently more than one gene involved in antibiotic resistance (12), we cannot rule out the possibility that *orfA* is part of the kirrothricin gene cluster.

The question remained how *orfA* exerts its negative influence on lipase production. It is unlikely that *lipA* transcription is affected by the transcription of *orfA*, since there is a terminator structure in front of *orfA*. This terminator functions at least in *E. coli*, since no lipase activity was detected in *E. coli* (pUPS6), in which *lipA* is in frame with *lacZ*. A more likely explanation of the negative influence of *orfA* on lipase production is that overexpression (gene dose effect) of *orfA* and the concomitant increased production of a transmembrane protein are deleterious for the cells. It is well known (although not much attention is given to this point in the literature) that overproduction of a membrane protein can affect cell growth and thus can exert a selective pressure for the loss of the plasmid, decrease in its copy number, or other events that lead to a reduction in the expression of the respective gene.

It is surprising that the 275-amino-acid *S. cinnamomeus* lipase, LipA, shares no sequence similarity with the available lipase sequences. The two *Streptomyces* lipase sequences published, the 310-amino-acid lipase of *Streptomyces* sp. strain M11 (42) and the 304-amino-acid lipase of *S. albus* (11), exhibit 82% sequence identity and contain two cysteine residues. Since the primary structure of *S. cinnamomeus* LipA differs from that of the two other *Streptomyces* lipases, we assume that the biochemical properties of LipA differ from those of the other two *Streptomyces* lipases. LipA also shows no sequence similarity to the sequences of the esterases of *S. scabies* and *S.*

diastatochromogenes, although these esterases contain six cysteine residues like LipA.

Sequence similarity to LipA (29% identity and 60% similarity within the first 104 N-terminal amino acids) was found only with the group II *Pseudomonas* lipases, with *P. cepacia* and *P. glumae* lipases as the prototypes (27). These two lipases are 33 kDa in size, have a broad substrate specificity, contain one disulfide bridge (two cysteine residues present), and require a helper protein, which acts as a molecular chaperone in assisting proper lipase folding (19, 20, 29). The corresponding helper protein genes are located immediately downstream of the lipase genes. The *Pseudomonas* lipases play an increasing role in biotechnology, since they are capable of hydrolysis of triglycerides, of transesterification, and of glycerolysis of fats and oils, leading to the production of certain monoglycerides that act as surfactants and are used as emulsifiers (40, 59).

The *S. cinnamomeus* lipase probably also produces toxic cell surfactants in the presence of olive oil, thus selecting clones in which the plasmid-encoded *lipA* is genetically inactivated or the plasmid is lost, as mentioned above.

A striking feature of the LipA produced in *S. lividans* and in *E. coli* was that the active form migrated in the *S. lividans* clone as a 50-kDa protein (very probably a dimer form) and in the *E. coli* clone as a 27-kDa active form. A 50-kDa form in a stained protein gel was also visible in the *E. coli* clone; however, it showed no activity, possibly because it is not correctly folded. The 27-kDa activity band indicates that LipA either is active as a monomer or can reassociate in vitro to form an active dimer.

Since LipA has sequence similarity to the *Pseudomonas* group II lipases, which require a chaperone for folding, and since only an active 50-kDa form of LipA is found in *S. cinnamomeus* and the *S. lividans* clone, it is possible that a chaperone is necessary for the correct folding of LipA in *Streptomyces*. The downstream sequence of *lipA*-localized *orfB* could be a candidate. However, it is unlikely that the *orfA* or *orfB* gene products aid in LipA folding or secretion, since OrfA has sequence similarity to proton-dependent transport proteins and OrfB shows, apart from the NAD and flavin adenine dinucleotide binding motif, no significant similarity to other proteins. This study is a basis for further analysis of the biochemical properties of LipA, especially the triglycerol synthesis, to study the dimerization reaction and secretion of the enzyme.

ACKNOWLEDGMENTS

We are very grateful to Volker Gnau for amino acid sequencing, Elisabeth Knorpp for photographic reproductions, and Karen A. Brune for editing the manuscript.

P.S. was supported by the KAAD. This work was supported by grants from the Deutsche Forschungsgemeinschaft (Go371/2-1, SFB323).

REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403–410.
2. **Ayora, S., P.-E. Lindgren, and F. Götz.** 1994. Biochemical properties of a novel metalloprotease from *Staphylococcus hyicus* subsp. *hyicus* involved in extracellular lipase processing. J. Bacteriol. **176:**3218–3223.
3. **Beck, E., G. Ludwig, E. A. Auerswald, B. Reiss, and H. Schaller.** 1982. Nucleotide sequence and exact localization of the neomycin phosphotransferase gene from transposon Tn5. Gene **19:**327–336.
4. **Bissonnette, L., S. Champetier, J.-P. Buisson, and P. Roy.** 1991. Characterization of the nonenzymatic chloramphenicol resistance (*cmlA*) gene of the integron Tn*1696*: similarity of the product to transmembrane transport proteins. J. Bacteriol. **173:**4493–4502.
5. **Blanc, V., K. Salah-Bey, M. Folcher, and C. J. Thompson.** 1995. Molecular characterization and transcriptional analysis of a multiple drug resistance gene cloned from the pristinamycin-producing organism, *Streptomyces prostinaespiralis*. Mol. Microbiol. **17:**989–999.
6. **Borgstroem, B.** 1988. Mode of action of tetrahydrolipstatin: a derivative of the naturally occurring lipase inhibitor lipstatin. Biochim. Biophys. Acta **962:**308–316.
7. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72:**248–254.
8. **Brady, L., A. M. Brzozowski, Z. S. Derewenda, E. Dodson, G. Dodson, S. Tolley, J. P. Turkenburg, L. Christiansen, B. Huge-Jensen, L. Norskov, and U. Menge.** 1990. A serine protease triad forms the catalytic centre of a triacylglycerol lipase. Nature **351:**491–494.
9. **Brenner, S.** 1988. The molecular evolution of genes and proteins: a tale of two serines. Nature **334:**528–530.
10. **Brune, K. A., and F. Götz.** 1992. Degradation of lipids by bacterial lipases, p. 243–266. *In* J. Winkelmann (ed.), Microbial degradation of natural products. VCH, Weinheim, Germany.
11. **Cruz, H., C. Pérez, E. Wellington, C. Castro, and L. Servín-González.** 1994. Sequence of the *Streptomyces albus* G lipase-encoding gene reveals the presence of a prokaryotic lipase family. Gene **144:**141–142.
12. **Cundliffe, E.** 1989. How antibiotic-producing organisms avoid suicide. Annu. Rev. Microbiol. **43:**227–233.
13. **Cygler, M., J. D. Schrag, J. L. Sussman, M. Harel, I. Silman, M. K. Gentry, and B. P. Doctor.** 1993. Relationship between sequence conservation and three-dimensional structure in a large family of esterases, lipases, and related proteins. Protein Sci. **2:**366–382.
14. **Dartois, V. A., A. Baulard, K. Schanck, and C. Colson.** 1992. Cloning, nucleotide sequence and expression of a lipase gene from *Bacillus subtilis* 168. Biochim. Biophys. Acta **1131:**253–260.
15. **Demleitner, G., and F. Götz.** 1994. Evidence for importance of the *Staphylococcus hyicus* lipase pro-peptide in lipase secretion, stability, and activity. FEMS Microbiol. Lett. **121:**189–198.
16. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
17. **Dower, W. J., J. F. Miller, and C. W. Ragsdaler.** 1988. High efficiency transformation of E. coli by high voltage electroporation. Nucleic Acids Res. **16:**6127–6145.
18. **Fernández-Moreno, M. A., J. L. Caballero, D. Hopwood, and F. Malpartida.** 1991. The *act* cluster contains regulatory and antibiotic export genes, direct targets for translational control by *bldA* tRNA gene of *Streptomyces*. Cell **66:**769–780.
19. **Frenken, L. G., A. de Groot, J. Tommassen, and C. T. Verrips.** 1993. Role of the *lipB* gene product in the folding of the secreted lipase of *Pseudomonas glumae*. Mol. Microbiol. **9:**591–599.
20. **Frenken, L. G., M. R. Egmond, A. M. Batenburg, J. W. Bos, C. Visser, and C. T. Verrips.** 1992. Cloning of the *Pseudomonas glumae* lipase gene and determination of the active site residues. Appl. Environ. Microbiol. **58:**3787–3791.
21. **Henderson, P. J. F., and M. C. J. Maiden.** 1990. Homologous sugar transport proteins in *Escherichia coli* and their relatives in both prokaryotes and eukaryotes. Philos. Trans. R. Soc. London **326:**391–410.
22. **Hochuli, E., E. Kupfer, R. Maurer, W. Meister, Y. Mercadal, and K. Schmidt.** 1987. Lipstatin, an inhibitor of pancreatic lipase, produced by *Streptomyces toxytricini*. II. Chemistry and structure elucidation. J. Antibiot. **40:**1086–1091.
23. **Hongo, E., M. Morimyo, K. Mita, I. Machida, H. Hama-Inaba, H. Tsuji, S. Ichimura, and Y. Noda.** 1994. The methyl viologen-resistance-encoding gene *smvA* of *Salmonella typhimurium*. Gene **148:**173–174.
24. **Hopwood, D. A., T. Kieser, K. F. Chater, C. J. Burton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Schrempf.** 1985. Genetic manipulation on *Streptomyces*. A laboratory manual. The John Innes Institute, Norwich, United Kingdom.
25. **Hunter, I. S.** 1985. Gene cloning in Streptomyces: molecular cloning. IRC Press, Oxford, United Kingdom.
26. **Iizumi, T., K. Nakamura, Y. Shimada, A. Sugihara, Y. Tominaga, and T. Fukase.** 1991. Cloning, nucleotide sequencing, and expression in *Escherichia coli* of a lipase and its activator genes from *Pseudomonas* sp. KWI-56. Agric. Biol. Chem. **55:**2349–2357.
27. **Jaeger, K.-E., S. Ransac, B. W. Dijkstra, C. Colson, M. van Heuvel, and O. Misset.** 1994. Bacterial lipases. FEMS Microbiol. Rev. **15:**29–63.
28. **Jäger, S., G. Demleitner, and F. Götz.** 1992. Lipase of *Staphylococcus hyicus*: analysis of the catalytic triad by site-directed mutagenesis. FEMS Microbiol. Lett. **100:**249–254.
29. **Jorgensen, S., K. W. Skow, and B. Diderichsen.** 1991. Cloning, sequence and expression of a lipase gene from *Pseudomonas cepacia*: lipase production in heterologous hosts requires two *Pseudomonas* genes. J. Bacteriol. **173:**559–567.
30. **Katz, E., C. J. Thompson, and D. A. Hopwood.** 1983. Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. J. Gen. Microbiol. **129:**2703–2714.
31. **Kitahara, M., M. Asano, H. Naganawa, K. Maeda, M. Hamada, T. Aoyagi, and H. Umezawa.** 1987. Valilacton, produced by actinomycetes. J. Antibiot. **40:**1647–1650.
32. **Kouker, G., and K.-E. Jaeger.** 1987. Specific and sensitive plate assay for bacterial lipases. Appl. Environ. Microbiol. **53:**211–213.

33. **Kupke, T., M. Lechner, G. Kaim, and F. Götz.** 1989. Improved purification and biochemical properties of phosphatidylinositol-specific phospholipase C from *Bacillus thuringiensis*. Eur. J. Biochem. **185:**151–155.
34. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
35. **Lechner, M., T. Kupke, S. Stefanovic, and F. Götz.** 1989. Cloning and sequencing of a gene from *Bacillus thuringiensis* encoding phosphatidylinositol-specific phospholipase C. Mol. Microbiol. **3:**621–626.
36. **Lesuisse, E., K. Schanck, and C. Colson.** 1993. Purification and preliminary characterization of the extracellular lipase of *Bacillus subtilis* 168, an extremely basic pH-tolerant enzyme. Eur. J. Biochem. **216:**155–160.
37. **Liebl, W., and F. Götz.** 1986. Lipase directed export of *Escherichia coli* β-lactamase in *Staphylococcus carnosus*. Mol. Gen. Genet. **204:**166–173.
38. **Mattern, S. G., M. E. Brawner, and J. Westpheling.** 1993. Identification of a complex operator for *galP1*, the glucose-sensitive, galactose-dependent promoter of the *Streptomyces* galactose operon. J. Bacteriol. **175:**1213–1220.
39. **Nikoleit, K., R. Rosenstein, H. M. Verheij, and F. Götz.** 1995. Comparative biochemical and molecular analysis of the *Staphylococcus hyicus*, *Staphylococcus aureus* and a hybrid lipase: indication for a C-terminal phospholipase domain. Eur. J. Biochem. **228:**732–738.
40. **Osada, K., K. Takahashi, and M. Hatano.** 1990. Polyunsaturated fatty acid glyceride synthesis by microbial lipases. J. Am. Oil Chem. Soc. **67:**921922.
41. **Pearson, W. R., and D. J. Lipmann.** 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85:**2444–2448.
42. **Pérez, C., K. Juaréz, E. Garcia-Castells, G. Sóberon, and L. Sérvin-González.** 1993. Cloning, characterization and expression in *Streptomyces lividans* 66 of an extracellular lipase-encoding gene from *Streptomyces* sp. M11. Gene **123:**109–114.
43. **Pigac, J., D. Hranueli, T. Smokyina, and M. Alacevic.** 1982. Optimal cultural conditions for handling *Streptomyces* protoplasts. Appl. Environ. Microbiol. **44:**1178–1186.
44. **Raymer, G., J. M. A. Willard, and J. L. Schottel.** 1990. Cloning, sequencing, and regulation of the expression of an extracellular esterase gene from plant pathogenic *Streptomyces scabies*. J. Bacteriol. **172:**7020–7026.
45. **Rouch, D. A., D. S. Cram, D. Diberardino, T. G. Littlejohn, and R. A. Skurray.** 1990. Efflux-mediated antiseptic resistance gene *qacA* from *Staphylococcus aureus*: common ancestry with tetracycline- and sugar-transport proteins. Mol. Microbiol. **4:**2051–2062.
46. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
47. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
48. **Scrutton, N. S., A. Berry, and R. N. Perham.** 1990. Redesign of the coenzyme specificity of a dehydrogenase by protein engineering. Nature **343:**38–43.
49. **Shirahama, T., T. Furumi, and M. Okanashi.** 1981. A modified method for regeneration of *Streptomyces* protoplasts. Agric. Biol. Chem. **45:**1271–1273.
50. **Simons, J.-W., H. Adams, R. C. Cox, N. Dekker, F. Götz, A. J. Slotboom, and H. M. Verheij.** 1996. The lipase from *Staphylococcus aureus*: expression in *Escherichia coli*, large-scale purification and comparison of substrate specificity to *Staphylococcus hyicus* lipase. Eur. J. Biochem. **242:**760–769.
51. **Strohl, W. R.** 1992. Compilation and analysis of DNA sequences associated with apparent streptomycete promotors. Nucleic Acids Res. **20:**961–974.
52. **Takiff, H. E., M. Cimino, M. C. Musso, T. Weisbrod, R. Martinez, M. B. Delgado, L. Salazar, B. R. Bloom, and W. R. J. Jacobs.** 1996. Efflux pump of the proton antiporter family confers low-level fluoroquinolone resistance in *Mycobacterium smegmatis*. Proc. Natl. Acad. Sci. USA **93:**362–366.
53. **Tesch, C., K. Nikoleit, V. Gnau, F. Götz, and C. Bormann.** 1996. Biochemical and molecular characterization of the extracellular esterase from *Streptomyces diastatochromogenes*. J. Bacteriol. **178:**1858–1865.
54. **Thein-Schranner, L., H. Zähner, H. U. Hoppe, I. Hummel, and A. Zeeck.** 1982. Metabolic products of microorganisms. Kirrothricin, a new member of the kirromycin group. J. Antibiot. **35:**948–956.
55. **van Oort, M. G., A. M. T. J. Deveer, R. Dijkman, M. L. Tjeenk, H. M. Verheij, G. H. de Haas, E. Wenzig, and F. Götz.** 1989. Purification and substrate specificity of *Staphylococcus hyicus* lipase. Biochemistry **28:**9278–9285.
56. **Vara, J. A., M. Lewundowska-Skarbek, Y.-G. Wang, S. Donadio, and C. R. Hutchison.** 1989. Cloning of genes governing the deoxysugar portion of the erytromycin biosynthetic pathway in *Saccharopolyspora erythraea* (*Streptomyces erythreus*). J. Bacteriol. **171:**5872–5881.
57. **Wei, Y., J. L. Schottel, U. Derewenda, L. Swenson, S. Patkar, and Z. S. Derewenda.** 1995. A novel variant of the catalytic triad in the *Streptomyces scabies* esterase. Nat. Struct. Biol. **2:**218–223.
58. **Weibel, E. K., P. Hadvary, E. Hochuli, E. Kupfer, and H. Lengsfeld.** 1987. Lipstatin, an inhibitor of pancreatic lipase, produced by *Streptomyces toxytricini*. I. Producing organism, fermentation, isolation and biological activity. J. Antibiot. **40:**1081–1085.
59. **Yang, B., W. J. Harper, K. L. Parkins, and J. Chen.** 1994. Screening of commercial lipases for production of mono and diacylglycerols from butteroil by enzymatic glycerolysis. Int. Dairy J. **4:**1–13.
60. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vector and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.