JOURNAL OF BACTERIOLOGY, Jan. 1991, p. 559–567
0021-9193/91/020559-09$02.00/0
Copyright © 1991, American Society for Microbiology
Vol. 173, No. 2

Cloning, Sequence, and Expression of a Lipase Gene from *Pseudomonas cepacia*: Lipase Production in Heterologous Hosts Requires Two *Pseudomonas* Genes

STEEN JØRGENSEN,* KIRSTEN W. SKOV, AND BØRGE DIDERICHSEN
Novo Nordisk, DK-2880 Bagsvaerd, Denmark

Received 12 July 1990/Accepted 12 October 1990

The *lipA* gene encoding an extracellular lipase from *Pseudomonas cepacia* was cloned and sequenced. Downstream from the lipase gene an open reading frame was identified, and the corresponding gene was named *limA*. *lipA* was well expressed only in the presence of *limA*. *limA* exerts its effect both in *cis* and in *trans* and therefore produces a diffusible gene product, presumably a protein of 344 amino acids. Replacement of the *lipA* expression signals (promoter, ribosome-binding site, and signal peptide-coding sequences) by heterologous signals from gram-positive bacteria still resulted in *limA*-dependent *lipA* expression in *Escherichia coli*, *Bacillus subtilis*, and *Streptomyces lividans*.

Extracellular lipases (triacylglycerol acylhydrolase [EC 3.1.1.3]) are produced by a variety of microorganisms (34), and the genes for several such lipases have been cloned. Lipase genes of bacterial origin obtained from *Staphylococcus hyicus* were cloned and expressed in *Staphylococcus carnosus* and *Escherichia coli* (14); genes from *Staphylococcus aureus* were cloned and expressed in *E. coli*, *Bacillus subtilis*, and *S. aureus* (22); genes from *Alcaligenes denitrificans* were cloned and expressed in *E. coli* (27); genes from *Pseudomonas aeruginosa* were cloned and expressed in *E. coli* and *P. aeruginosa* (27, 39); and genes from *Pseudomonas fragi* were cloned and expressed in *E. coli* (2, 21). DNA sequences are available for the lipase genes from *S. hyicus* (15), *S. aureus* (23), and *P. fragi* (2, 21). These cloned bacterial lipase genes were in all cases directly expressed in the host organism used for cloning.

We have cloned and sequenced a lipase gene, *lipA*, from a strain of *Pseudomonas cepacia* and have investigated its expression in *E. coli*, *B. subtilis*, and *Streptomyces lividans*. Surprisingly, good expression of *lipA* in these hosts depends on the presence of a second gene, *limA*, which in *P. cepacia* is linked to *lipA*.

MATERIALS AND METHODS

Bacterial strains. The following strains were used: *P. cepacia* DSM 3959, *E. coli* NM539 (12) and SJ2 (10), *B. subtilis* DN1885 (10), and *Streptomyces lividans* TK24 (18).

Plasmids. *E. coli* plasmids used were pUC18 (40), pUC19 (40), and pACYC177 (6). *B. subtilis* plasmid pDN1528 is a derivative of pUB110 (15) harboring a chloramphenicol resistance gene and carrying a 2.3-kbp *Hin*dIII-*Sph*I fragment containing the alpha-amylase gene from a derivative of *Bacillus licheniformis* ATCC 9789. *Streptomyces* plasmids were pIJ702 (18), pIJ2002 (5), and pIJ4642. The last is a positive selection vector for *E. coli* into which DNA fragments can be inserted between two phage fd terminators by using *Bam*HI. It is derived from pIJ666 (18a, 20) and was obtained from T. Kieser.

Plasmid constructions. Plasmids derived directly from pSJ150 by subcloning or deletion are described in Fig. 1.

(i) *limA* deletion plasmids. pSJ252 (Fig. 1) was digested with *Sac*I and *Bam*HI and treated with exonuclease III (Exo III), thus creating deletions extending from the right-hand end of the cloned DNA. Deletion derivatives pSJ316, pSJ318, pSJ319, pSJ320, and pSJ323 contain deletions at positions 1460, 1759, 1970, 2110, and 2197, respectively. The *Pseudomonas* DNA insert was reversed with respect to the *lacZ* promoter by cloning *Eco*RI-*Sph*I fragments from the deletion derivatives into *Eco*RI-*Sph*I-digested pUC18, resulting in pSJ653, pSJ655, pSJ657, pSJ659, and pSJ661, respectively. Then *Pvu*II fragments from these plasmids were inserted into *Hin*dII-digested pACYC177 to give plasmids pSJ676, pSJ678, pSJ680, pSJ682, and pSJ684, respectively.

(ii) *limA*-containing pACYC177 derivatives. The *limA*-containing *Sph*I insert in pSJ377 and pSJ378 (Fig. 1) was excised together with the *lacZ* promoter as a 1.5-kbp *Pvu*II fragment and inserted into *Hin*dII-digested pACYC177, giving pSJ622 and pSJ624, respectively.

(iii) *amyL-lipA* fusion plasmids. pSJ150 was digested with *Bam*HI, and the resulting 3.6-kbp fragment was religated, giving pSJ243. A synthetic oligonucleotide adapter containing the sequences extending from a *Pst*I site near the C-terminal end of the *B. licheniformis* alpha-amylase (*amyL*) signal peptide (32) to the processing site followed by the sequences extending from the mature *Pseudomonas* lipase N terminus to the *Mlu*I site closely downstream was prepared (KFN454 plus KFN457; sequence 5′-GCAGCGGCCGCA GCTGGCTACGCGGCGA-3′). This adapter was inserted into plasmid pSJ243 digested with *Pst*I and *Mlu*I, forming pSJ385. pSJ385 was digested with *Eco*RI, and the resulting 3.1-kbp fragment was ligated to the 2.0-kbp *Eco*RI fragment from pSJ150, forming pSJ424. pSJ424 was digested with *Cla*I and partially with *Pst*I, and the 0.9-kbp *Cla*I-*Pst*I fragment was ligated to the 3.1-kbp *Cla*I-*Pst*I fragment from pDN1528, forming pSJ493 and pSJ494 (Fig. 2). pSJ416 (Fig. 2) was constructed by ligating simultaneously the 0.5-kbp *Bam*HI-*Pst*I fragment from pSJ385 and the 1.9-kbp *Eco*RI-*Bam*HI fragment from pSJ150 to the 3.1-kbp *Pst*I-*Eco*RI fragment from pDN1528.

(iv) *dagA-lipA* fusion plasmids. A synthetic oligonucleotide

* Corresponding author.

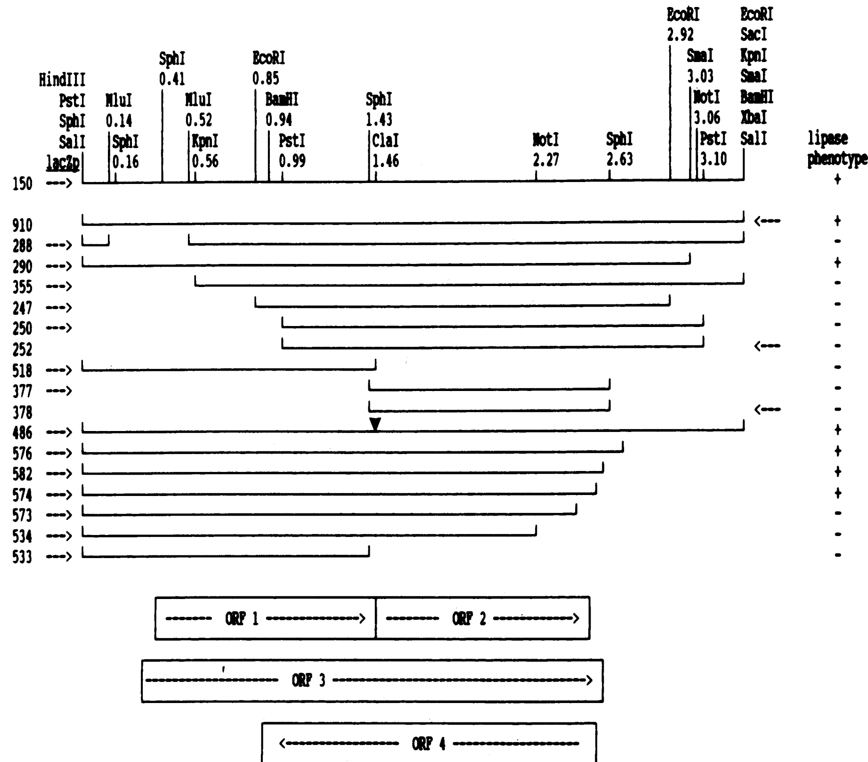

FIG. 1. Restriction map of the insert on pSJ150 and deletion derivatives. Symbols: – – – – →, direction of transcription from the *lacZ* promoter on the pUC vector plasmids: ▼ , small insertion; + or –, phenotype of *E. coli* SJ2 containing the plasmid (halo formation on tributyrine plates). pSJ910 was obtained by insertion of the 3.3-kbp *Sac*I-*Hin*dIII fragment from pSJ150 into *Sac*I-*Hin*dIII-digested pUC18. pSJ288 and pSJ290 were constructed by deletion from pSJ150 of the 0.35-kbp *Mlu*I fragment and the 0.32-kbp *Sma*I fragment, respectively. pSJ355, pSJ247, pSJ250, and pSJ252 contain the 2.7-kbp *Kpn*I fragment, the 2.06-kbp *Eco*RI fragment, or the 2.10-kbp *Pst*I fragment from pSJ150 inserted into pUC19 digested with the corresponding enzyme. pSJ518 contains the 1.5-kbp *Hin*dIII-*Cla*I fragment from pSJ150 inserted into *Hin*dIII-*Acc*I-digested pUC19. pSJ377 and pSJ378 contain the 1.20-kbp *Sph*I fragment from pSJ150 inserted into *Sph*I-digested pUC19. pSJ486 is pSJ150 modified by insertion of an 8-bp *Bgl*II linker into the *Cla*I site filled in with Klenow fragment of DNA polymerase I. pSJ573, pSJ574, pSJ576, and pSJ582 were obtained by Exo III deletion of *Sma*I-*Sac*I-digested pSJ150, and pSJ533 and pSJ534 were obtained by Exo III deletion of *Not*I-*Sac*I-digested pSJ150. They all contain a *Bgl*II site at the deletion endpoint, since *Bgl*II linkers were added prior to ligation. The positions of the four open reading frames deduced from the DNA sequence given in Fig. 3 are indicated.

adapter containing the sequences extending from an *Ava*II site present near the C-terminal end of the *Streptomyces coelicolor* agarase (*dagA*) signal peptide (5) to the processing site followed by the sequences extending from the mature *Pseudomonas* lipase N terminus past the *Mlu*I site closely downstream and terminating in a *Xho*I end was prepared (KFN587 plus KFN590; sequence 5′-GTCCCGCACCCGC CGCTCATGCCGCAGCTGGCTACGCGGCGACGCGTC 3′). This adapter, together with an 880-bp *Hin*dIII-*Ava*II fragment containing the *dagA* promoter region purified from pIJ2002, was ligated to *Hin*dIII-*Sal*I-digested pUC19, forming plasmid pSJ604. The 0.9-kbp *Mlu*I-*Hin*dIII fragment from pSJ604 was then ligated either to the 4.7-kbp *Hin*dIII-*Mlu*I fragment from pSJ574 (Fig. 1), forming pSJ616, or to the 5.5-kbp *Hin*dIII-*Mlu*I fragment from pSJ486 (Fig. 1), forming pSJ619. pIJ702 was digested with *Bgl*II and ligated either to *Bgl*II-digested pSJ616, forming pSJ669, or to *Bgl*II-digested pSJ619, forming pSJ671 (Fig. 2). These plasmids were constructed in *E. coli*.

(v) *E. coli* plasmid containing *amyL-lipA* fusion. pSJ909 (Fig. 2) was constructed by ligating *Bgl*II-digested pSJ494 into *Bam*HI-digested pIJ4642.

Lambda DNA. The phage lambda cloning vector used was EMBL4 (12).

Media. For growth of *E. coli* and *B. subtilis*, LB (25) and 2% agar were used for solid media. TY medium (10) was used for liquid media. Lambda-infected cells were plated in 3 ml of soft agar (LB with 0.6% agar and 10 mM $MgSO_4$) poured on top of standard LB plates. Protoplasts of *Streptomyces* species were plated in soft-agar overlayers on R2YE plates as described elsewhere (18). Regenerated transformants were propagated on agar medium of the following composition (per liter): peptone (Difco), 6 g; trypsin-digested casein, 4 g; Difco yeast extract, 3 g; meat extract, 1.5 g; glucose, 1.0 g; and agar, 2.4 g. For use in lipase screening, 10 ml of tributyrine and 1 g of gum arabic per liter were added to the melted agar medium and emulsified with an Ultra Turrax emulsifier. The shake flask medium used for *B. subtilis* was prepared as a suspension of 100 g of potato flour, 50 g of barley flour, 0.1 g of BAN 5000 SKB (an alpha-amylase supplied by Novo Nordisk A/S which is inactivated during autoclaving of the medium), 10 g of sodium caseinate, 20 g of soya bean extract, 9 g of $Na_2HPO_4 \cdot 12H_2O$, and 0.1 g of pluronic acid per liter final volume. When appropriate, media were supplemented with ampicillin (100 or 200 μg/ml), kanamycin (30 μg/ml), chloramphenicol (6 μg/ml), or thiostrepton (500 μg/ml in soft agar overlayers, 50 μg/ml in solid media, or 5 μg/ml in liquid media).

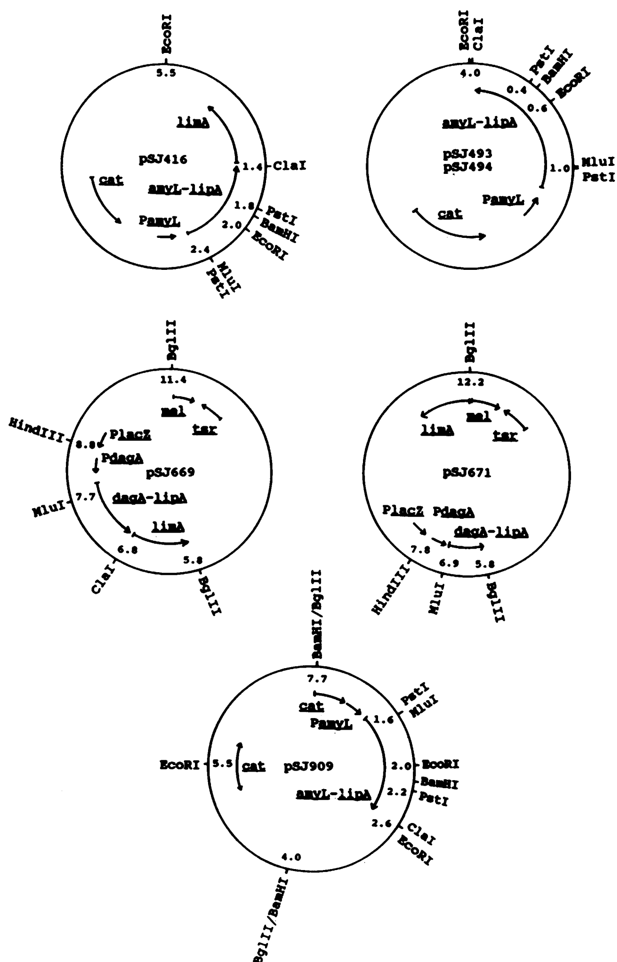

FIG. 2. Maps of plasmids pSJ416, pSJ493, pSJ494, pSJ669, pSJ671, and pSJ909. *cat* is the chloramphenicol acetylase gene of plasmid pC194 of *S. aureus*, *tsr* is the thiostrepton resistance gene from *Streptomyces azureus*, and *mel* is the tyrosinase gene from *Streptomyces antibioticus*. P*amyL* indicates the promoter for the alpha-amylase gene of *B. licheniformis*, P*lacZ* is the β-galactosidase promoter of pUC19, and P*dagA* is the promoter for the *Streptomyces coelicolor* agarase gene. *amyL-lipA* indicates the alpha-amylase–lipase fusion gene, and *dagA-lipA* indicates the agarase-lipase fusion gene.

Transformation and phage infection. Competent cells of *E. coli* and *B. subtilis* and protoplasts of *Streptomyces lividans* were prepared and transformed by established procedures (18, 24, 41). Phage lambda DNA was packaged in vitro by using Packagene extract (Promega), and packaged phage were plated on recipient cells as described by the supplier.

DNA manipulations. Chromosomal DNA was prepared by phenol extraction (41). Plasmid DNAs from all strains were prepared by alkaline phenol extraction (19). Phage lambda DNA was prepared from 10 ml of liquid cultures (25). Restriction, ligation, end filling, and exonuclease treatment were performed with enzymes from New England BioLabs and under conditions recommended by the supplier. Exo III was used for generating deletions (17). DNA fragments for construction of the gene bank and recombinant plasmids were isolated from agarose gels by using DEAE-cellulose paper (11).

Oligonucleotides. Primers and linkers were obtained from New England BioLabs or synthesized on an Applied Biosystems DNA synthesizer and purified by polyacrylamide gel electrophoresis before use.

DNA sequencing. DNA sequencing was performed by the dideoxy chain termination method (30), with ^{35}S-dATP (DuPont NEN; NEG034H; >1,000 Ci/mmol) as the radioactive label and denatured plasmid DNAs as templates. Sequencing was carried out either as described by Hattori and Sakaki (16) or by using Sequenase (United States Biochemical Corp.). The sequences of both strands were determined by using a combination of subclones of restriction fragments, deletions from either end of cloned fragments done with Exo III, and synthetic oligonucleotide primers.

Colony hybridizations. Colonies were transferred to Whatman 541 paper filters, lysed, and immobilized (13). The filters were hybridized with ^{32}P-labeled oligonucleotides. Hybridization and washing were done at 42°C and were followed by autoradiography at −80°C for 1.5 h with an intensifier screen.

Southern blotting analysis. Transfer of DNA was performed by electroblotting as described in the instruction manual for GeneScreen Plus hybridization transfer membranes (Dupont NEN Research Products). pSJ247, ^{32}P-labeled by nick translation with the Nick Translation Kit from Amersham, was used as probe. Hybridizations were performed at 50°C and were followed by washing at 60°C (25).

Sequence analysis. Analyses of DNA and protein sequences were performed on a personal computer using the PC/GENE program, version 5.16, from GENOFIT SA. Database searches were performed on a VAX computer using the GCG program package (9).

Western blots (immunoblots). Antibodies raised in rabbits against lipase purified from *P. cepacia* were kindly supplied by M. Schülein of Novo Nordisk. Proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis were transferred to nitro-cellulose membranes by electroblotting, which was followed by immunodetection (4).

Lipase assay. Lipase was measured by a pH-stat method using an emulsion of tributyrine as substrate. One unit is the amount of enzyme which liberates 1 μmol of titratable butyric acid per min under the following conditions: 30.0°C, pH 7.0, 1 g of gum arabic per liter as emulsifier, and 50 ml of tributyrine per liter as substrate.

Nucleotide sequence accession number. The nucleotide sequence data reported here will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under accession number M58494.

RESULTS

Cloning of the lipase gene. Genomic DNA from *P. cepacia* DSM 3959 was partially digested with *Sau*IIIA, and DNA fragments of between 9 and 23 kbp were ligated to *Bam*HI-digested lambda EMBL4 DNA. The ligation mixture was packaged into phage particles and plated on *E. coli* NM539, and phages from the resulting 2,500 plaques were harvested. Phages from this stock were plated on NM539 in soft agar containing 1% glycerol tributyrate emulsion, and clear plaques, indicating hydrolysis of the turbid emulsion, were obtained at a frequency of about 1/1,000. DNA preparations from three such phages were identical as judged by restriction digests. The lipase gene, named *lipA*, was subcloned from one of these phages onto pUC19 by using a partial *Sal*I

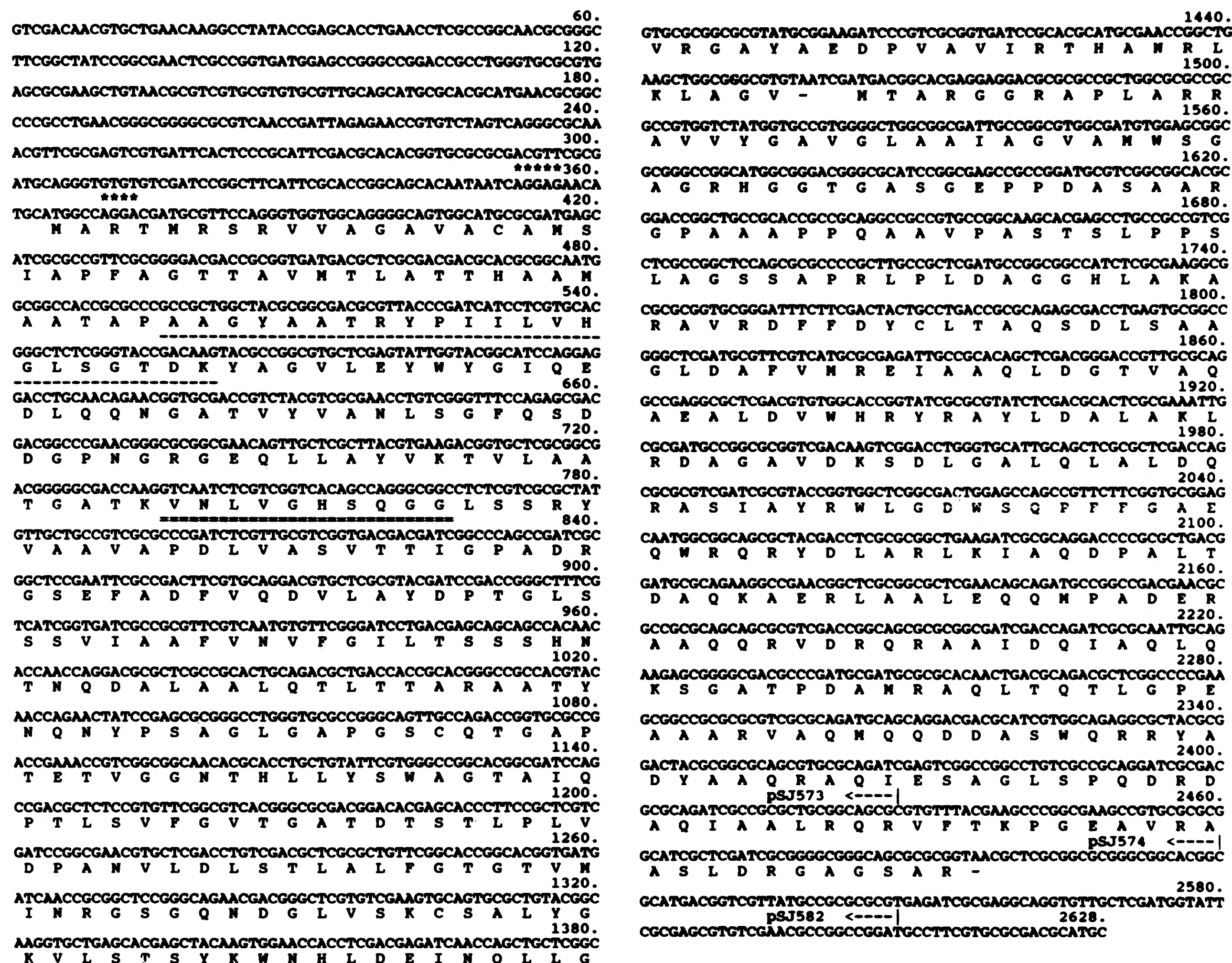

FIG. 3. DNA sequence of the *lipA* region and deduced amino acid sequences of the encoded proteins. Two putative ribosome-binding site sequences are indicated above the sequence (*), the N-terminal amino acid sequence of mature lipase is underscored, the substrate-binding site region is double underscored, and deletion endpoints for plasmids pSJ573, pSJ574, and pSJ582 are indicated by arrows above the sequence.

digestion. Transformants of *E. coli* SJ2 were screened on tributyrine plates, and one lipase-producing recombinant was kept as SJ150. This strain contained a plasmid, pSJ150, of approximately 6 kbp. The restriction map of the insert on pSJ150 is shown in Fig. 1. It was confirmed by Southern hybridization that the 2.0-kbp *Eco*RI fragment internal to the cloned DNA on pSJ150 was found in *Eco*RI-digested genomic DNA from DSM 3959 (data not shown).

Deletion analysis of pSJ150. *lipA* on pSJ150 was localized by subcloning and deletion with Exo III. Some of the resulting plasmids and their phenotypes are shown in Fig. 1. We conclude that the region necessary for lipase production extends to either side of the *Mlu*I and *Not*I sites at 0.52 and 2.27 kbp, respectively. However, insertion of a *Bgl*II linker in the *Cla*I site at 1.46 kbp did not abolish lipase expression (pSJ486, Fig. 1). Since lipase is expressed both from pSJ150 and from pSJ910, in which the 3.3-kbp insert is reversed (Fig. 1), an active promoter on the cloned fragment must transcribe the lipase gene. The halos formed around colonies containing pSJ910 were smaller than those formed around colonies containing pSJ150, indicating that the *lacZ* promoter on pSJ150 contributes to transcription of the lipase gene. However, isopropyl-β-D-thiogalactopyranoside had no effect on halo formation and was omitted in the following experiments.

DNA sequence. The sequence of *lipA* on pSJ150 was determined. It extends from the *Sal*I site at 0 kbp to the *Sph*I site at 2.63 kbp and is given in Fig. 3. The sequenced DNA contained 69.3% G+C base pairs, close to the 67.4% characteristic for *P. cepacia* (29).

Four major open reading frames, ORF1 to ORF4 in Fig. 1, can be identified from the sequence. Each contains several putative initiation codons. ORF3 and ORF4 are not essential for lipase expression for the following reasons. First, the linker insertion in the *Cla*I site, present in pSJ486, disrupts both of these reading frames without affecting lipase expression. Second, deletion plasmid pSJ574 (Fig. 1) lacks a small part of both ORF3 and ORF4 but still confers the lipase-positive phenotype.

ORF1 corresponds to the structural gene for the lipase, *lipA*, as indicated by the following observations. (i) It encodes an amino acid sequence (VNLVGHSQGG, posi-

tions 736 to 765 in the DNA sequence) which is very well conserved among lipases and is believed to participate in substrate binding (1). (ii) It encodes a sequence identical to the N-terminal amino acid sequence determined from purified lipase from *P. cepacia* (underscored in Fig. 3), and the expected size of the mature protein (320 amino acids) corresponds well with the molecular weight of *P. cepacia* lipase (30a). (iii) The encoded protein has high homology to a lipase from *P. fragi* (2). The two lipases can be aligned, introducing eight gaps in total, to show identity at 104 positions and similarity at 46 positions (PC/GENE program PALIGN).

Upstream from the N terminus of the mature lipase, a sequence resembling a typical signal peptide extends for 40 or 44 amino acids. The ATG codon positioned 44 amino acids upstream is the most likely start codon, since the sequence AGGAG found 7 bp further upstream resembles a typical ribosome-binding site (31). The ATG 40 amino acids upstream is preceded by the sequence AGGA but with less-than-ideal spacing (only 2 bp), making it a less likely initiation site. The putative signal peptide is unusually long and is unusual also in having a proline at the −1 position (35, 36).

lipA is transcribed from a promoter on the cloned fragment. We were, however, unable to identify any sequences upstream from *lipA* resembling either the *E. coli* sigma 70 consensus sequence (26) or known *Pseudomonas* promoters (8).

The *lipA* gene terminates just upstream from the *Cla*I site at 1.46 kbp. However, it was found from the initial deletion analysis of pSJ150 that the region necessary for efficient lipase expression extended further downstream, to around 2.5 kbp (Fig. 1). We tentatively conclude that a gene, hereafter called *limA*, for lipase modulator, participates in the expression of active lipase and is situated in the 1-kbp region downstream from the *lipA* gene. Open reading frame ORF2 may correspond to *limA*. The stop codon of ORF2 is retained on the smallest deletion plasmid still expressing lipase (pSJ574), whereas 22 C-terminal amino acids from ORF2 are removed on the lipase-negative deletion plasmid pSJ573 (Fig. 3).

That both ORF1 and ORF2 correspond to protein-coding genes is substantiated by analysis of their codon usage, which shows a marked preference for codons having G or C in their third position, as would be expected for genes from organisms with a high genomic G+C content (3, 38).

***limA* is *trans* acting.** Deletion of part or all of *limA* from pSJ150 abolished the lipase-positive phenotype (Fig. 1). To examine whether the positive effect of *limA* was exerted in *trans* also, the *limA* gene was transcribed in either of the possible directions from a *lac* promoter on pACYC-derived plasmids pSJ622 and pSJ624.

Strain SJ518 is *E. coli* SJ2 containing the pUC19-derived plasmid pSJ518 (Fig. 1), in which the cloned DNA extends to the *Cla*I site immediately downstream from the *lipA* gene. SJ518 is lipase negative. It was transformed with either pSJ622 or pSJ624, selecting for both kanamycin and ampicillin resistance, and transformants were streaked on plates containing glycerol tributyrate emulsion. Large halos formed around colonies containing both pSJ518 and pSJ622, in which the *lacZ* promoter transcribes ORF2 in the proper direction. No halos, however, formed around colonies containing both pSJ518 and pSJ624, in which ORF2 is not expected to be expressed (Fig. 4).

Expression of the *limA* gene in *trans* to the *lipA* gene thus confers a lipase-positive phenotype on the host strain. The effect was seen only with pSJ622, as expected if ORF2 encodes the diffusible effector molecule.

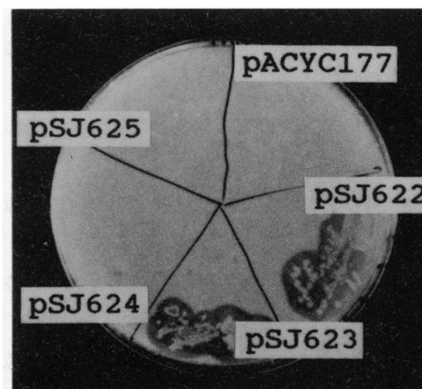

FIG. 4. Effect of *limA* in *trans* to *lipA*. *E. coli* SJ518 was transformed with pSJ622 (=pSJ623), pSJ624 (=pSJ625), or pACYC177 as described in the text, and transformants were streaked on a tributyrine plate. Incubation was at 37°C for 2 days.

Exact localization of *limA* gene. The functional 5′ end of the *limA* gene was determined by using the *trans* effect of *limA*, since its expression could be scored by the lipase-positive phenotype of strain SJ518 containing a *limA*-positive plasmid.

Deletions had been made in the left end of the *Pst*I-*Pst*I fragment (0.99 to 3.10 kbp, Fig. 1). pACYC-derived plasmids containing, downstream from a *lacZ* promoter, fragments extending from position 1461, 1760, 1971, 2111, or 2198 to the *Pst*I site at about position 3100 were constructed (pSJ676 to pSJ684) and introduced into strain SJ518. Only pSJ676, containing DNA starting at position 1461, conferred a lipase-positive phenotype on strain SJ518, whereas the others gave lipase-negative phenotypes. This result localized the 5′ end of the *limA* gene between positions 1461 and 1760. However, this region contains several possible start codons in ORF2, and therefore a more detailed analysis was required.

limA is contained on plasmid pSJ377. The sequence of pSJ377 from the *lacZ* start codon across the *Sph*I site and 87 bp into the inserted DNA is given in Fig. 5. In this plasmid, the last 10 codons of the *lipA* gene are fused in frame to the *lacZ* start. Deletions in both directions from the *Cla*I site were made by using Exo III. To obtain deletions confined to the previously determined region, positions 1461 to 1760, only plasmids hybridizing to both a pUC19 reverse sequencing primer (hybridizing just upstream from the *Hin*dIII site) and a synthetic oligonucleotide (hybridizing at positions 1763 to 1780) in a colony hybridization were picked and sequenced. The sequences of five deletion derivatives are given in Fig. 5. Several longer deletions were also obtained, as well as insertions of 1 or 2 bp at the *Cla*I site (not shown). To determine the effect of these modifications on *limA* expression, the entire constructs (including the *lacZ* promoter) were transferred, by using *Pvu*II, from pUC19 into *Hin*dII-digested pACYC177. The resulting plasmids were introduced into SJ518, and their phenotypes were scored (indicated in Fig. 5). The following observations were made. (i) Insertion of 1 or 2 bp at the *Cla*I site did not abolish *limA* expression. (ii) Deletion of the TAA stop codon for the lipase gene from pSJ377 did not abolish *limA* expression (pSJ716). (iii) The slightly larger deletion of 11 bp (pSJ703) abolished *limA* expression. So did all the larger deletions (e.g., in

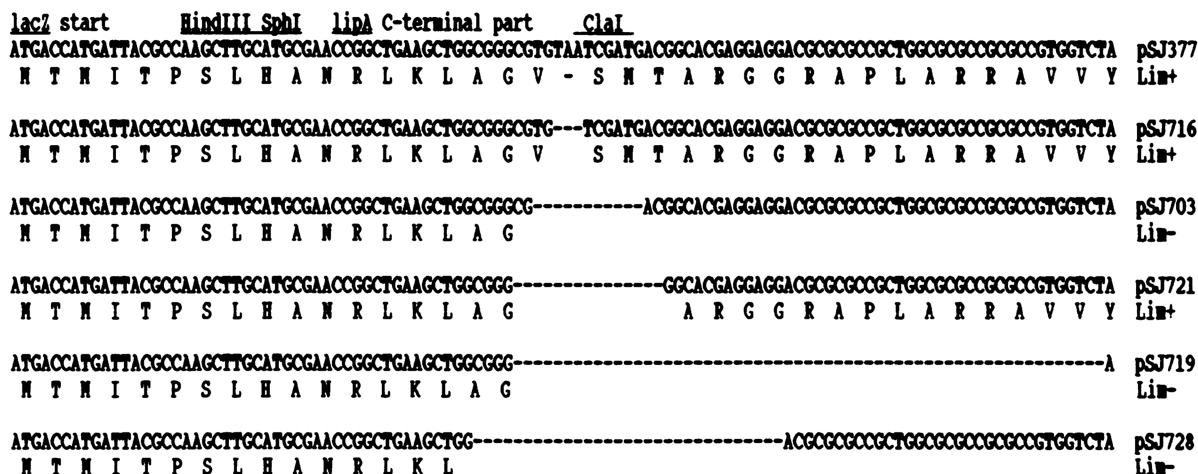

FIG. 5. Sequence of pSJ377 from the *lacZ* start codon into the *limA*-containing *SphI* fragment, with sequences of five deletion derivatives and their phenotypes indicated. The broken line indicates the extent of deletion in each plasmid.

pSJ728, with 31 bp deleted), except for those in pSJ721 (see below).

These experiments locate the 5′ end of the *limA* gene to the region deleted on pSJ703 and on pSJ728. The only putative start codon in this region is the ATG overlapping the *ClaI* site, i.e., the first of the possible start codons for ORF2. There is no obvious ribosome-binding site in front of this ATG, however. Further evidence that the *limA* product is a protein encoded by the open reading frame identified above was obtained from the phenotype conferred by pSJ721. The deletion in pSJ721 was slightly larger than that in pSJ703, which conferred a lipase-negative phenotype. Despite this, pSJ721 conferred a lipase-positive phenotype. This observation may be explained by the formation of a fusion protein, retaining the lipase-modulating activity, from pSJ721. It is apparent from Fig. 5 that the pSJ721 deletion removed the *limA* start codon, but the rest of *limA* was fused in frame to a 17-codon region consisting of nine N-terminal codons from the pUC19 *lacZ* gene plus eight C-terminal codons from the *lipA* gene.

Lim protein. The *limA* open reading frame could encode a protein of 344 amino acids with a molecular weight of 36,500. The N-terminal amino acid sequence of this predicted protein, called Lim, resembles a signal peptide, with a putative processing site between alanine 34 and glycine 35 (Fig. 3) (PC-Gene program PSIGNAL). Alternatively, Lim could be a membrane-associated protein with amino acids 14 to 34 forming a transmembrane helix (PC-Gene, programs RAOARGOS and HELIXMEM). No protein homologous to Lim has been identified in the National Biomedical Research Foundation protein sequence database (release 24.0).

Expression of *lipA* in *B. subtilis*. Expression of *lipA* in *B. subtilis* was attempted by replacing the native expression signals with those from a well-expressed *Bacillus* gene. The promoter, ribosome-binding site, and signal peptide-coding region from a *B. licheniformis* alpha-amylase gene were fused in frame to DNA encoding the mature part of the lipase. Two different constructions were made: pSJ493, in which the *Pseudomonas* DNA present in the plasmids extends only to the *ClaI* site immediately downstream from the *lipA* gene, and pSJ416, in which the *Pseudomonas* DNA extends to an *EcoRI* site downstream from the *limA* gene (Fig. 2). These plasmids were introduced into *B. subtilis* DN1885, and lipase production was measured in shake flask cultures with the growth medium described in Materials and Methods. Strains with pSJ416, which contained the *limA* gene in addition to the modified *lipA* gene, produced 30 to 40 U of lipase per ml, whereas yields from strains with pSJ493, containing no *limA* gene, were 1 to 2 U/ml. The background level for strain DN1885 is 0 to 5 U/ml.

Lipase protein in the culture broth from the same shake flasks was also analyzed by Western blotting (Fig. 6). This indicated that lipase with a molecular weight corresponding to that of the expected processed form and comigrating with lipase purified from *P. cepacia* was synthesized from the modified *lipA* gene by *B. subtilis*. The lipase protein was observed in culture broth from strains containing either construction but, as a comparison of lane 8 with lanes 1 and 2 shows, in at least 10-fold-smaller amounts when the *limA* gene was deleted from the plasmid. Since there is no

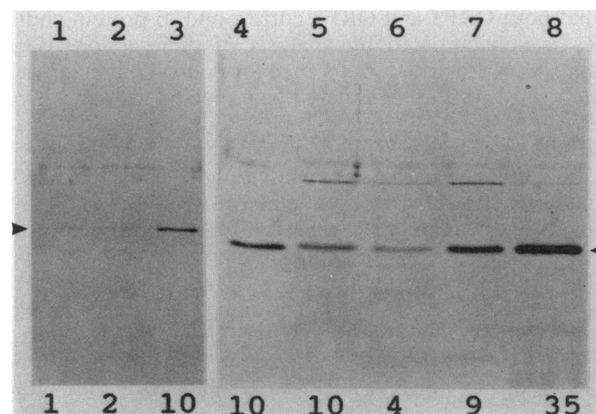

FIG. 6. Western blots of samples of total culture broth from shake flasks with *B. subtilis* transformants, using the growth medium described in Materials and Methods. The figure below each lane indicates lipase activity (units per milliliter), measured as described in Materials and Methods. The position of the lipase protein is indicated by arrowheads. Lanes 1 and 2: DN1885 containing pSJ493 grown for 4 days at 37°C; lanes 3 and 4, purified lipase from *P. cepacia*; lanes 5, 6, and 7, DN1885 containing pSJ416, with various growth conditions and times of sampling; lane 8, DN1885 containing pSJ416 grown for 4 days at 37°C.

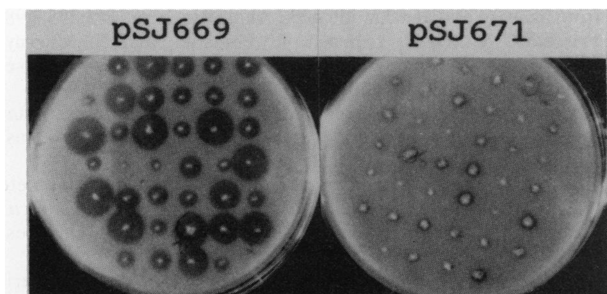

FIG. 7. Effect of *limA* expression on lipase production in *Streptomyces lividans*. Strain TK24 was transformed with the plasmids indicated, and transformants were transferred to plates with glycerol tributyrate emulsion. Incubation was at 37°C for 2 days.

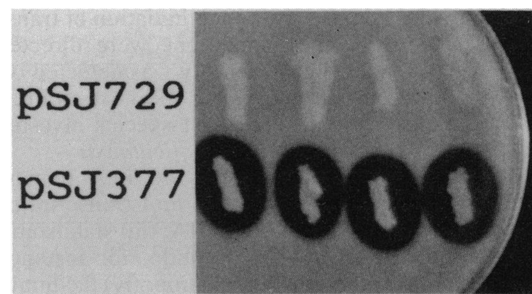

FIG. 8. Effect of *limA* expression on lipase production from the *amyL-lipA* fusion gene in *E. coli*. SJ909 (*E. coli* SJ2 containing pSJ909) was transformed with the plasmids indicated, and transformants were transferred to a plate with glycerol tributyrate emulsion. Incubation was at 37°C for 7 days.

discrepancy between activity and protein measurements, it is unlikely that significant amounts of inactive lipase are accumulated in the absence of *limA*. The expression of *limA* from pSJ416 is probably not very efficient, as *limA* lacks any obvious ribosome-binding site for initiation of translation in *B. subtilis*. Some translational coupling between *lipA* and *limA* may, however, take place. We conclude that expression of *lipA* by heterologous expression signals in *B. subtilis* is *limA* dependent.

Expression of *lipA* in *S. lividans*. The *lipA* gene was expressed in *Streptomyces lividans* by fusing the promoter, ribosome-binding site, and signal peptide-coding region from a *Streptomyces coelicolor* agarase gene in frame to DNA encoding the mature part of the lipase. Two different *E. coli-Streptomyces lividans* shuttle vectors were constructed. In pSJ669, the modified *lipA* gene is followed by the *limA* gene exactly as on the original clone of *lipA* and *limA*, whereas pSJ671 contains the modified *lipA* gene and the *limA* gene separated from each other by 5.8 kbp of pIJ702 *Streptomyces* vector DNA, with no known promoters transcribing *limA* (Fig. 2). The plasmids constructed in *E. coli* were introduced into *Streptomyces lividans* TK24, and the transformants were transferred to plates containing glycerol tributyrate. Large halos formed around transformants with pSJ669, but only small halos formed around transformants with pSJ771 (Fig. 7). Expression of the *limA* gene thus also has a positive effect on production of lipase from the modified *lipA* gene in *S. lividans*.

Expression of *lipA* from heterologous expression signals in *E. coli* is *limA* dependent. The fusion between the *B. licheniformis* alpha-amylase expression signals and the *lipA* gene contained on the *B. subtilis* plasmid pSJ494 (identical to pSJ493 described above) was transferred to *E. coli* by cloning of the entire pSJ494 into *E. coli* vector pIJ4642, to give pSJ909 (Fig. 2). This construction contains the modified *lipA* gene but not the *limA* gene. *E. coli* SJ909 (containing pSJ909) is lipase negative, as indicated by the absence of any halos around the colonies on plates with glycerol tributyrate.

Strain SJ909 was then transformed either with *limA*-expressing plasmid pSJ377 (described above) or with pSJ729, a deletion derivative of pSJ377 lacking about 600 bp from the 5′ terminus of the *limA* gene, thus abolishing *limA* expression. Transformants of SJ909 containing pSJ377 were surrounded by clear halos on plates with glycerol tributyrate, whereas no halos were seen around those containing pSJ729 (Fig. 8).

The *trans*-acting effect of *limA* is thus seen in *E. coli* not only when lipase expression is directed by the signals of the *lipA* gene (i.e., promoter, ribosome-binding site, and signal peptide coding sequence), but also when these signals are replaced by heterologous, gram-positive expression signals.

DISCUSSION

This report describes the molecular cloning, DNA sequence, and expression in heterologous hosts of *lipA*, the gene for an extracellular lipase from *P. cepacia*. The lipase is synthesized as a precursor with a 44-amino-acid signal peptide. The mature lipase of 320 amino acids contains the amino acid sequence characteristic for the substrate-binding site of lipases (1) and shows extensive sequence homology to a lipase from *P. fragi* (2).

We have discovered that the *lipA* gene alone was insufficient to confer a lipase-positive phenotype on heterologous host strains and have identified a second gene, *limA*, which participates in lipase production. The *limA* gene is positioned immediately downstream from the *lipA* gene and is transcribed in the same direction, probably by the *lipA* promoter. The lipase-positive phenotype is observed only when both the *lipA* and *limA* genes are expressed in the same host strain. This effect of *limA* is observed also in *trans* when *lipA* and *limA* are carried on different plasmids in the same cell. The *limA* gene product is most likely a protein of 344 amino acid residues, LimA. No homologies between the LimA protein and proteins described in sequence databases were found.

The described phenomenon involving *lipA* and *limA* appears unique among lipases. It is particularly surprising considering that the lipase gene from *P. fragi*, which shows an extensive sequence homology with the *lipA* gene, is expressed in *E. coli* in the absence of other *Pseudomonas* genes and the *P. fragi* DNA sequence gives no indication of a *lim*-like gene (2).

The question of how *limA* participates in lipase production naturally arises. The LimA protein cannot be part of the active lipase enzyme, since the lipase purified to homogeneity from *P. cepacia* retained full activity. Furthermore, both N-terminal amino acid sequence and total amino acid composition confirm that active lipase consists of only the polypeptide species encoded by *lipA*.

The data presented exclude the possibility that *limA* regulates expression of *lipA* by affecting initiation of either transcription or translation of the *lipA* gene. Several plasmids were constructed, in which all *P. cepacia* DNA upstream from that encoding the mature part of the lipase protein was replaced with totally unrelated sequences direct-

ing expression of the lipase. Although initiation of transcription and translation of the lipase gene were directed by heterologous sequences, lipase activity was detected only if the *limA* gene was also expressed in the host strain. This applies to expression in all three host species investigated (*E. coli*, *B. subtilis*, and *Streptomyces lividans*).

The following hypothetical effects of *limA* remain: (i) stimulation of elongation of either the *lipA* transcript or the LipA protein; (ii) stabilization of mRNA; (iii) stabilization of the nascent or mature *lipA* polypeptide, for instance by protection against proteolytic degradation; (iv) facilitation of secretion, for instance by maintaining the lipase in a secretion-compatible conformation or by participating in secretion by interaction with the membrane. Analogies to this last possibility can be found for other secreted proteins of gram-negative origin, e.g., the hemolysin of *E. coli* (28, 37), the leucotoxin of *Pasteurella haemolytica* (33), and the pullulanase of *Klebsiella pneumoniae* (7). These systems have in common the need for specific secretion-mediating proteins to ensure proper export of the extracellular product. The finding that *limA* encodes a signal peptide-like sequence indicates a possible association between the LimA protein and the cell membrane and supports the hypothesis that *limA* in some way participates in secretion of the lipase.

ACKNOWLEDGMENTS

We thank Kjeld Norris and Fanny Norris for synthesis of oligonucleotides, Martin Schülein and Lars Thim for lipase purification and N-terminal sequence determination, and Svend Aage Hansen for performing Western blotting experiments.

REFERENCES

1. **Antonian, E.** 1988. Recent advances in the purification, characterization and structure determination of lipases. Lipids **23:** 1101–1106.
2. **Aoyama, S., N. Yoshida, and S. Inouye.** 1988. Cloning, sequencing and expression of the lipase gene from *Pseudomonas fragi* IFO-12049 in *E. coli*. FEBS Lett. **242:**36–40.
3. **Bibb, M. J., P. R. Findlay, and M. W. Johnson.** 1984. The relationship between base composition and codon usage in bacterial genes and its use for the simple and reliable identification of protein-coding sequences. Gene **30:**157–166.
4. **Bjerrum, O. J., and N. H. Heegaard.** 1987. Handbook of immunoblotting of proteins. CRC Press, Boca Raton, Fla.
5. **Buttner, M. J., I. M. Fearnley, and M. J. Bibb.** 1987. The agarase gene (*dag*A) of *Streptomyces coelicolor* A3(2): nucleotide sequence and transcriptional analysis. Mol. Gen. Genet. **209:**101–109.
6. **Chang, A. C. Y., and S. N. Cohen.** 1978. Construction and amplification of amplifiable multicopy DNA cloning vehicles derived from the p15A cryptic miniplasmid. J. Bacteriol. **134:** 1141–1156.
7. **d'Enfert, C., A. Ryter, and A. P. Pugsley.** 1987. Cloning and expression in *Escherichia coli* of the *Klebsiella pneumoniae* genes for production, surface localization and secretion of the lipoprotein pullulanase. EMBO J. **6:**3531–3538.
8. **Deretic, V., W. M. Konyecsni, C. D. Mohr, D. W. Martin, and N. S. Hibler.** 1989. Common denominators of promoter control in *Pseudomonas* and other bacteria. Bio/Technology **7:**1249–1254.
9. **Devereux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12:**387–395.
10. **Diderichsen, B., U. Wedsted, L. Hedegaard, B. R. Jensen, and C. Sjøholm.** 1990. Cloning of *aldB*, which encodes α-acetolactate decarboxylase, an exoenzyme from *Bacillus brevis*. J. Bacteriol. **172:**4315–4321.
11. **Dretzen, G., M. Bellard, P. Sassone-Corsi, and P. Chambon.** 1981. A reliable method for the recovery of DNA fragments from agarose and acrylamide gels. Anal. Biochem. **112:**295–298.
12. **Frischauf, A.-M., H. Lehrach, A. Poutstka, and N. Murray.** 1983. Lambda replacement vectors carrying polylinker sequences. J. Mol. Biol. **170:**827–842.
13. **Gergen, J. P., R. H. Stern, and P. C. Wensink.** 1979. Filter replicas and permanent collections of recombinant DNA plasmids. Nucleic Acids Res. **7:**2115–2136.
14. **Götz, F., F. Popp, E. Korn, and K. H. Schleifer.** 1985. Complete nucleotide sequence of the lipase gene from *Staphylococcus hyicus* cloned in *Staphylococcus carnosus*. Nucleic Acids Res. **13:**5895–5906.
15. **Gryczan, T. J., S. Contente, and D. Dubnau.** 1978. Characterization of *Staphylococcus aureus* plasmids introduced by transformation into *Bacillus subtilis*. J. Bacteriol. **134:**318–329.
16. **Hattori, M., and Y. Sakaki.** 1986. Dideoxy sequencing method using denatured plasmid templates. Anal. Biochem. **152:**232–238.
17. **Henikoff, S.** 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene **28:**351–359.
18. **Hopwood, D. A., M. J. Bibb, K. F. Chater, T. Kieser, C. J. Bruton, H. M. Kieser, D. J. Lydiate, C. P. Smith, J. M. Ward, and H. Schrempf.** 1985. Genetic manipulation of *Streptomyces*. A laboratory manual. The John Innes Foundation, Norwich, England.

18a. **Kieser, T.** Personal communication.

19. **Kieser, T.** 1984. Factors affecting the isolation of CCC DNA from *Streptomyces lividans* and *Escherichia coli*. Plasmid **12:** 19–36.
20. **Kieser, T., and R. E. Melton.** 1988. Plasmid pIJ699, a multicopy positive-selection vector for streptomyces. Gene **65:**83–91.
21. **Kugimiya, W., Y. Otani, Y. Hashimoto, and Y. Takagi.** 1986. Molecular cloning and nucleotide sequence of the lipase gene from *Pseudomonas fragi*. Biochem. Biophys. Res. Commun. **141:**185–190.
22. **Lee, C. Y., and J. J. Iandolo.** 1985. Mechanism of bacteriophage conversion of lipase activity in *Staphylococcus aureus*. J. Bacteriol. **164:**288–293.
23. **Lee, C. Y., and J. J. Iandolo.** 1986. Lysogenic conversion of staphylococcal lipase is caused by insertion of the bacteriophage L54a genome into the lipase structural gene. J. Bacteriol. **166:**385–391.
24. **Mandel, A., and A. Higa.** 1970. Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. **53:**159–162.
25. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
26. **McClure, W. R.** 1985. Mechanism and control of transcription initiation in procaryotes. Annu. Rev. Biochem. **54:**171–204.
27. **Odera, M., K. Takeuchi, and A. Toh-e.** 1986. Molecular cloning of lipase genes from *Alcaligenes denitrificans* and their expression in *Escherichia coli*. J. Ferment. Technol. **64:**363–371.
28. **Oropeza-Wekerle, R. L., E. Müller, P. Kern, R. Meyermann, and W. Goebel.** 1989. Synthesis, inactivation, and localization of extracellular and intracellular *Escherichia coli* hemolysins. J. Bacteriol. **171:**2783–2788.
29. **Palleroni, N. J.** 1984. *Pseudomonas cepacia*, p. 175–176. *In* N. R. Kreig and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
30. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.

30a. **Schülein, M.** Personal communication.

31. **Shine, J., and L. Dalgarno.** 1974. The 3′-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. USA **71:**1342–1346.
32. **Stephens, M. A., S. A. Ortlepp, J. F. Ollington, and D. J. McConnell.** 1984. Nucleotide sequence of the 5′ region of the *Bacillus licheniformis* alpha-amylase gene: comparison with the *B. amyloliquefaciens* gene. J. Bacteriol. **158:**369–372.
33. **Strathdee, C. A., and R. Y. C. Lo.** 1989. Cloning, nucleotide sequence, and characterization of genes encoding the secretion

function of the *Pasteurella haemolytica* leukotoxin determinant. J. Bacteriol. **171**:916–928.

34. **Sztajer, H., and I. Maliszewska.** 1988. Production of exogenous lipases by bacteria, fungi, and actinomycetes. Enzyme Microb. Technol. **10**:492–497.
35. **von Heijne, G.** 1985. Signal sequences. The limits of variation. J. Mol. Biol. **184**:99–105.
36. **von Heijne, G.** 1986. A new method for predicting signal sequence cleavage sites. Nucleic Acids Res. **14**:4683–4690.
37. **Wagner, W., M. Vogel, and W. Goebel.** 1983. Transport of hemolysin across the outer membrane of *Escherichia coli* requires two functions. J. Bacteriol. **154**:200–210.
38. **West, S. E. H., and B. H. Iglewski.** 1988. Codon usage in *Pseudomonas aeruginosa*. Nucleic Acids Res. **16**:9323–9335.
39. **Wohlfarth, S., and U. K. Winkler.** 1988. Chromosomal mapping and cloning of the lipase gene of *Pseudomonas aeruginosa*. J. Gen. Microbiol. **134**:433–440.
40. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**:103–119.
41. **Yasbin, R. E., G. A. Wilson, and F. E. Young.** 1975. Transformation and transfection in lysogenic strains of *Bacillus subtilis*: evidence for selective induction of prophage in competent cells. J. Bacteriol. **121**:296–304.