Journal of General Microbiology (1992), **138**, 1325–1335. *Printed in Great Britain*

Molecular genetics of the extracellular lipase of *Pseudomonas aeruginosa* PAO1

SUSANNE WOHLFARTH,* CHRISTINE HOESCHE,† CORINA STRUNK and ULRICH K. WINKLER

Ruhr-Universität Bochum, Lehrstuhl für Biologie der Mikroorganismen, D-4630 Bochum, FRG

(*Received 10 December 1991; revised 27 February 1992; accepted 16 March 1992*)

The structural gene (*lipA*) coding for the extracellular lipase of *Pseudomonas aeruginosa* PAO1 has been cloned on plasmid pSW118. Nucleotide sequence analysis revealed a gene of 936 bp. *lipA* codes for a proenzyme of 311 amino acids including a leader sequence of 26 amino acids. The mature protein was predicted to have a M_r of 30134, an isoelectric point of 5·6, and a consensus sequence (IGHSHGG) typical of lipases. Furthermore it is highly homologous (>60%) to other lipases from various pseudomonads. The *lipA* gene failed to hybridize detectably with genomic DNA from other *Pseudomonas* species except *P. alcaligenes*, even under relaxed stringency. Located 220 bp downstream of the *lipA* gene, is an open reading frame (ORF2, *lipH*) which encodes a hydrophilic protein (283 amino acids; M_r 33587) that shows some homology to the *limA* gene product of *P. cepacia*. In complementation tests of lipase-defective mutants, *lipH* was shown to be necessary for expression of active extracellular lipase in *P. aeruginosa* PAO1.

Introduction

Lipases are triacylglycerol acylhydrolases (EC 3.1.1.3) that preferentially hydrolyse emulsified triglycerides. In organic solvents, lipases are also able to catalyse reverse reactions, e.g. the synthesis of esters, and transesterifications. The reaction mechanism postulated for the catalytic activities of a lipase involves an amino acid triad either of Ser-His-Asp (Winkler *et al.*, 1990; Brady *et al.*, 1990) or of Ser-His-Gln (Schrag *et al.*, 1991). To test this hypothesis, the amino acid sequences and the three-dimensional structures of further lipases have to be determined.

During the last few years, much progress has been made by investigating lipase genes from bacteria, especially from pseudomonads. Nucleotide sequences of lipase genes are already known for the following Gram-negative bacterial species: *P. fragi* IFO-3458 (Kugimiya *et al.*, 1986), *P. fragi* IFO-12049 (Aoyama *et al.*, 1988), *P. cepacia* (Jorgensen *et al.*, 1991), *P. fluorescens* SIK W1 (Chung *et al.*, 1991), *P. glumae* (Frenken *et al.*, 1991), *Pseudomonas* nov. sp. 109 (Ihara *et al.*, 1991), *Pseudomonas* sp. (Cox *et al.*, 1991), *Pseudomonas* sp. M-12-33 (Nakanishi *et al.*, 1991), *Pseudomonas* sp. (Nishioka *et al.*, 1991), *P. aeruginosa* PAO1 (this paper) and *Moraxella* sp. TA144 (Feller *et al.*, 1990, 1991). Furthermore, lipase genes from two Gram-positive species have been published: *Staphylococcus hyicus* (Götz *et al.*, 1985) and *S. aureus* (Lee & Iandolo, 1986).

The lipase proteins of two different strains of *P. aeruginosa* have been purified to homogeneity and have been characterized biochemically. The lipases of *P. aeruginosa* PAC1R (Stuer *et al.*, 1986; Jaeger *et al.*, 1991, and in press) and of *P. aeruginosa* EF2 (Gilbert *et al.*, 1991) both have an apparent M_r of 29000 and the mature proteins have the same N-terminal sequences (STYTQT-KYPIV...). The isoelectric points are 5·8 (*P. aeruginosa* PAC1R) and 4·9 (*P. aeruginosa* EF2). There is a strong association of the enzyme with lipopolysaccharides. The overall amino acid composition of the mature protein is known for the lipase of *P. aeruginosa* PAC1R. Analysis of two more lipase proteins purified from *Pseudomonas* sp. (Nishioka *et al.*, 1991) and from *Pseudomonas* nov. sp. 109 (Ihara *et al.*, 1991) also revealed an apparent M_r of 29000 and the same N-terminal sequence for the mature protein as mentioned above. An isoelectric point of 5·3 was measured for *Pseudomonas* nov. sp. 109.

The structural gene for the lipase of *P. aeruginosa* PAO1 has been mapped on the chromosome at about

* Author for correspondence.

† Present address: Ruhr-Universität Bochum, Institut für Physiologische Chemie, D-4630 Bochum, FRG.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession numbers X63390 (*lipA*) and X63391 (*lipH*).

57 min (old map) or 39 min (map according to O'Hoy & Krishnapillai, 1987) and it has been cloned on a 3·1 kb *Sal*I fragment (Wohlfarth & Winkler, 1988).

This paper reports the nucleotide sequence of the lipase gene (*lipA*) of *P. aeruginosa* PAO1 in order to compare the deduced amino acid sequence with those of other lipases and to identify conserved domains. In this connection, the lipase gene of *P. aeruginosa* PAO1 has been used as a DNA probe to search for homologous sequences in the chromosomal DNA of other pseudomonads. Moreover, the expression of the lipase gene has been studied. Finally we report on an open reading frame (ORF2, *lipH*) downstream of the structural gene of the lipase. The function of the gene product is unknown, but it seems to be involved in the formation of an enzymically active extracellular lipase.

Some of the results are included in European Patent no. 0 334 462 A1 (Andreoli *et al.*, 1989).

Methods

Bacteria, plasmids and phage. The following wild-type strains of *Pseudomonas aeruginosa* were used: PAO1 (Holloway *et al.*, 1979); FRD2 (D. Ohman, Berkeley, USA); ATCC 9027, ATCC 27853, Habs 02, Habs 12, DE-27, mucoid CF_1/M_1, CF_2/M_1, CF_3/M_1 (all from the stock culture collection of the institute, Bochum, FRG). *P. fluorescens* AFT36, *P. maltophilia* DSM 50170, *P. putida*, *P. stutzeri* AS 70 and *Escherichia coli* K12 were from the same source. *P. alcaligenes* DSM 50342, *P. cepacia* DSM 50181 and *P. fragi* DSM 3456 were purchased from Deutsche Sammlung von Mikroorganismen, Braunschweig, FRG. Besides these lipase-producing pseudomonads, two *lip* mutants of *P. aeruginosa* PAO1 (Wohlfarth & Winkler, 1988) were used: 29-1 (*met-9020 catA1 puuA1 lip1*) and 6-1 (*met-9020 catA1 puuA1 lip2*). *E. coli* JM109 (*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1λ⁻Δ(lac-proAB)* (*F'traD36 proAB lacI^q ZΔM15*) from Yanisch-Perron *et al.* (1985) was utilized as a cloning host.

For cloning and sequencing experiments plasmid vectors pBluescript II SK (Stratagene), pKT248 (Bagdasarian *et al.*, 1981), pUC19 (Yanisch-Perron *et al.*, 1985), pUC19Ps [this study: OriV from pRC354 (Chen *et al.*, 1987) inserted into *Pst*I restriction site of vector pUC19] and phage M13mp19 (Yanisch-Perron *et al.*, 1985) were used. Fig. 1 summarizes recombinant plasmids derived from pSW1 (Wohlfarth & Winkler, 1988) and pSW112. pCH1 was constructed to clone the lipase gene of *P. alcaligenes* DSM 50342.

Media and growth conditions. Strains of *Pseudomonas* were grown in nutrient broth (NB; Oxoid) with aeration at 37 °C. To select for plasmids, streptomycin sulphate (50 μg ml^{-1}), chloramphenicol (20 μg ml^{-1}) or carbenicillin (50 μg ml^{-1}) was added to the medium. *E. coli* was grown in Luria broth (LB) (Sambrook *et al.*, 1989), in some cases supplemented with ampicillin (50 μg ml^{-1}). IPTG (0·33 mM) and X-Gal (0·03%) were used to detect recombinant plasmids where appropriate. The cloned lipase gene was induced in *E. coli* using 1 mM-IPTG. To grow *E. coli* for transformation, 20 mM-Mg^{2+} (equal amounts of $MgSO_4$ and $MgCl_2$) was added to the broth medium. Agar plates comprised LB or NB solidified with 1·5 % (w/v) agar. M13 phages were propagated in LB or on LB agar plates overlaid with soft agar (LB with 0·5 % agar) containing IPTG and X-Gal as above. To distinguish between Lip^+ and Lip^- clones, a plate assay using calcium triolein (CT) agar was used (Wohlfarth & Winkler, 1988).

Transformation and phage infection. E. coli was transformed by an altered $CaCl_2$ method (Sambrook *et al.*, 1989). After growing *E. coli* in the presence of 20 mM-Mg^{2+} washed cells were suspended in 40 mM-$MnCl_2$, 100 mM-$CaCl_2$ and 50 mM-RbCl. For transformation of *P. aeruginosa*, early exponential growth phase cells were washed twice with 150 mM-$MgCl_2$. They were held at 4 °C for 18 h to induce competence (Wohlfarth & Winkler, 1988). Derivatives of phage M13 were propagated according to Sambrook *et al.* (1989).

DNA manipulations. Molecular genetic methods (e.g. isolation and purification of plasmid DNA and single stranded phage DNA) were done according to Sambrook *et al.* (1989). Chromosomal DNA was prepared by the method of Marmur (1961). Restriction enzymes and ligase were used as recommended by the suppliers (Gibco/BRL or Boehringer).

DNA sequencing and sequence analysis. Two strategies were used to obtain DNA sequences. (i) Single strand sequencing after shotgun cloning in M13mp19 of random fragments obtained by sonication (Deininger, 1983). Sequencing of these clones was performed by the dideoxy-nucleotide chain termination method of Sanger *et al.* (1977) using ^{35}S-dATP (0·37 MBq, Amersham) and the Klenow fragment of DNA polymerase. To prevent sequence compressions, 7-deaza-dGTP was used instead of dGTP. Sequence data were analysed on a Cyber 855 using the program of Staden (1980). (ii) Double strand sequencing of DNA fragments obtained by restriction endonuclease cleavage or by exonuclease III/mung bean deletions (Henikoff, 1984). Alkaline- or heat-denatured plasmid DNA was directly sequenced incorporating either ^{35}S-dATP or using fluorescently labelled universal or reversed M13-primer. The T7 Deaza-Sequencing Kit or AutoRead-Sequencing Kit (both from Pharmacia) were used. Non-radioactive sequences were generated by using Pharmacia's Automated Laser Fluorescent DNA Sequencer.

Nucleotide and deduced amino acid sequences were analysed using the following programs: Staden (Amersham), DNASIS v. 5·02 and PROSIS v. 6·00 (both Hitachi Software Engineering).

Southern and colony hybridization. Transfer of DNA was performed by capillary blotting onto Gene Screen Hybridization Transfer Membrane (NEN). Bacterial colonies were lysed and denatured on Colony/Plaque Screen Membranes (NEN) as recommended by the supplier. DNA probes were labelled with ^{32}P-dATP either by nick translation (Sambrook *et al.*, 1989) or by using the Random Primers Labelling Kit (Gibco/BRL). Hybridization and washing conditions were as described by NEN. The Digoxigenin DNA-Labelling System (Boehringer) was used for non-radioactive Southern analysis. Stringent conditions were obtained by hybridization and washing temperature of 60–65 °C. Relaxed conditions were obtained by lowering temperature to 45 °C.

Enzyme assay. Lipase activity was measured spectrophotometrically using *p*-nitrophenyl palmitate as substrate (Wohlfarth & Winkler, 1988).

Results

Cloning the lipase gene (lipA)

Plasmid pSW1 was previously shown to complement lipase-defective mutants 29-1 and 6-1 of *P. aeruginosa* PAO1 (Wohlfarth & Winkler, 1988). This plasmid carries 3 *Sal*I fragments cloned in pKT248. It was subjected to a detailed molecular genetic analysis (Fig. 1).

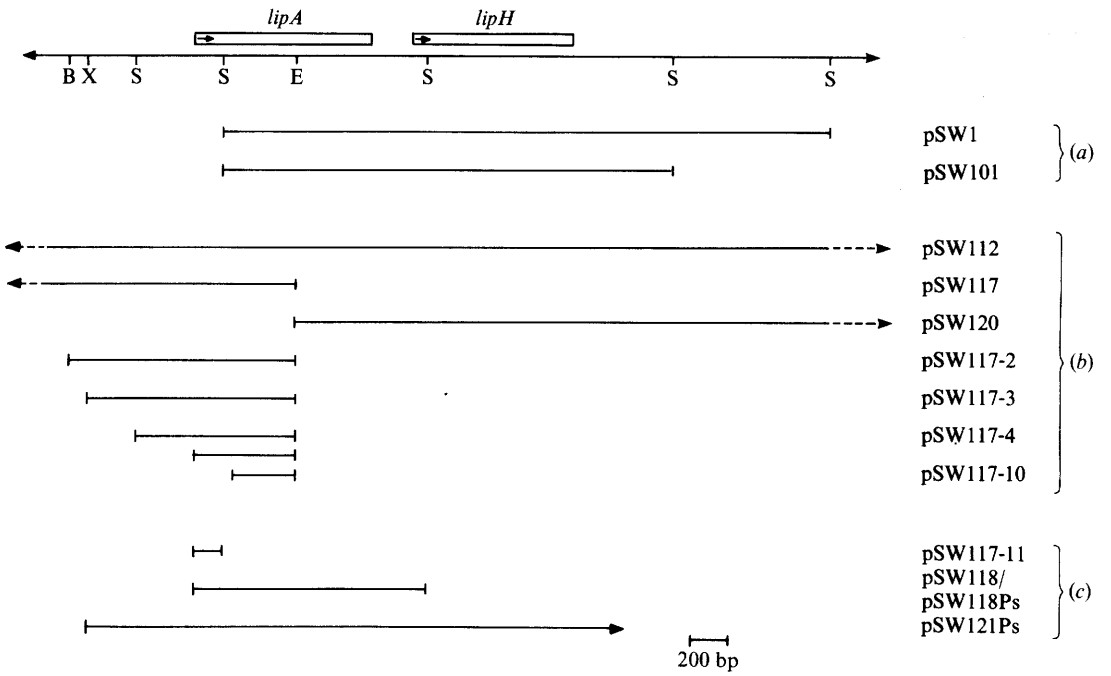

Fig. 1. Cloning and subcloning of the lipase gene from *P. aeruginosa* PAO1 and plasmid derivatives (pSW) used in this study. (*a*) Subcloning of pSW1: clones without N-terminus of the lipase gene. Vector: pKT248. (*b*) Cloning of the intact lipase gene using 1·0 kb *Sal*I fragment of pSW101 as a probe. Subcloning of pSW112; vector: pUC19. (*c*) Construction of clone pSW118 containing the intact lipase gene. Vectors: pBluescript SK II/pUC19Ps. Line drawings of inserts are to scale. Restriction sites are indicated by capital letters B: *Bam*HI; E, *Eco*RI; S, *Sal*I; X, *Xho*I. Genes *lipA* and *lipH* are included as boxes; arrows within indicate direction of transcription.

Two *Sal*I fragments (1·0–1·3 kb) were inserted into the *Sal*I restriction site within the chloramphenicol resistance gene of broad host range vector pKT248 (= pSW101). This plasmid also fully complemented both of the lipase-defective mutants. However, neither of these fragments alone expressed complementary activity. Sequence analysis (see below) revealed that the 5′ part of the lipase gene was missing from clone pSW101. The 1·0 kb *Sal*I fragment, which carries 85 % of the gene, was subsequently used as a probe to clone the intact gene.

Genomic DNA from *P. aeruginosa* PAO1 was digested with restriction endonuclease *Pvu*II, for which there is no cleavage site within the *lip* DNA in pSW101. Southern blot analysis showed a hybridizing fragment of about 20 kb. Fragments of approximately that size (15–25 kb) were isolated from an agarose gel, ligated into *Sma*I site of pUC19 and transformed into *E. coli* JM109. Twenty colonies out of about 800 recombinant clones gave positive hybridization signals. One plasmid having an insert of 15·3 kb was named pSW112 and was further analysed. It consisted of 12 *Sal*I fragments, including the 1·0 kb and 1·3 kb fragments present in pSW101 (verified by Southern hybridization, data not shown).

Restriction enzyme *Eco*RI, which cuts within the 1·0 kb *Sal*I fragment of the lipase gene, was used for subcloning into pUC19. Two clones were selected: pSW120 (insert: 3·5 kb) carrying the 3′ part and pSW117 (insert: 4·5 kb) carrying the 5′ part of the lipase gene, respectively. The *lip* DNA in pSW117 was then subcloned on a 1·2 kb *Eco*RI/*Bam*HI fragment into pUC19 cleaved with the same enzymes. The resulting plasmid pSW117-2 was further reduced in size by subcloning an *Eco*RI/*Xho*I fragment. The ends of this fragment were made blunt before ligation into *Sma*I site of pUC19 to form plasmid pSW117-3. The insert of pSW117-3 was subjected to progressive exonuclease digestion (Henikoff, 1984) starting from the *Bam*HI restriction site within the vector directly adjacent to the filled-in *Xho*I site of the insert. Plasmids pSW117-4 to pSW117-10 generated by this method were used for nucleotide sequencing.

Finally, a plasmid was constructed which carried the intact lipase gene *lipA* (Fig. 1). First, the 5′ part of *lipA* was cloned into pBluescript II SK forming plasmid pSW117–11, by cutting out a 0·15 kb *Sal*I/*Eco*RI fragment of plasmid pSW117-8 (the *Sal*I site comes from the lipase gene and the *Eco*RI site from the vector; Figs 1 and 2). The main part of the lipase gene (3′ part) came from plasmid pSW101 by ligating the 1·0 kb *Sal*I fragment into the *Sal*I site of plasmid pSW117–11, forming pSW118. The correct orientation of the 1·0 kb fragment (Fig. 1) to form an intact lipase gene was

```
1       10        20        30        40        50        60        70        80        90       100
GTCGACCATTTCAGCCTGTTTTGCTCGCAAAACGACGCCGCGGGCGTGCGCTACCGCACACTCCGTCGCTGGGCGTTGTGCGGGGAAGATTCAAACGAGC
      110       120       130       140       150       160       170       180       190       200
GTTTCGCGCCGTAACAACCCGCTCTCTTCCGCTCTGCCACGCAGGTTATGACCGGCCGCCAGGAAGCCGCGGATTTCCTGGCCTGGAGGAAAAAAGCCGA
      210       220       230       240       250       260       270       280       290       300
AGCTGGCACGGTTCCTGGCGCAAGGGACAGCGAAGCGGTTCTCCCGGAAGGATTCGGGCGATGGCTGGCAGGACGCGCCCCTCGGCCCCATCAACCTGAG
      310       320       330       340       350       360       370
****  ↓ORF1
ATGAGAACAAC ATG AAG AAG AAG TCT CTG CTC CCC CTC GGC CTG GCC ATC GGT CTC GCC TCT CTC GCT GCC AGC CCT
            M   K   K   K   S   L   L   P   L   G   L   A   I   G   L   A   S   L   A   A   S   P

380       390       400       410       420       430       440       450
CTG ATC CAG GCC AGC ACC TAC ACC CAG ACC AAA TAC CCC ATC GTG CTG GCC CAC GGC ATG CTC GGC TTC GAC AAC
L   I   Q   A   S   T   Y   T   Q   T   K   Y   P   I   V   L   A   H   G   M   L   G   F   D   N

      460       470       480       490       500       510       520
ATC CTC GGG GTC GAC TAC TGG TTC GGC ATT CCC AGC GCC TTG CGC CGT GAC GGT GCC CAG GTC TAC GTC ACC GAA
I   L   G   V   D   Y   W   F   G   I   P   S   A   L   R   R   D   G   A   Q   V   Y   V   T   E

530       540       550       560       570       580       590       600
GTC AGC CAG TTG GAC ACC TCG GAA GTC CGC GGC GAG CAG TTG CTG CAA CAG GTG GAG GAA ATC GTC GCC CTC AGC
V   S   Q   L   D   T   S   E   V   R   G   E   Q   L   L   Q   Q   V   E   E   I   V   A   L   S

      610       620       630       640       650       660       670
GGC CAG CCC AAG GTC AAC CTG ATC GGC CAC AGC CAC GGC GGG CCG ACC ATC CGC TAC GTC GCC GCC GTA CGT CCC
G   Q   P   K   V   N   L   I   G   H   S   H   G   G   P   T   I   R   Y   V   A   A   V   R   P

680       690       700       710       720       730       740       750
GAC CTG ATC GCT TCC GCC ACC AGC GTC GGC GCC CCG CAC AAG GGT TCG GAC ACC GCC GAC TTC CTG CGC CAG ATC
D   L   I   A   S   A   T   S   V   G   A   P   H   K   G   S   D   T   A   D   F   L   R   Q   I

      760       770       780       790       800       810       820
CCA CCG GGT TCG GCC GGC GAG GCA GTC CTC TCC GGG CTG GTC AAC AGC CTC GGC GCG CTG ATC AGC TTC CTT TCC
P   P   G   S   A   G   E   A   V   L   S   G   L   V   N   S   L   G   A   L   I   S   F   L   S

830       840       850       860       870       880       890       900
AGC GGC AGC ACC GGT ACG CAG AAT TCA CTG GGC TCG CTG GAG TCG CTG AAC AGC GAG GGT GCC GCG CGC TTC AAC
S   G   S   T   G   T   Q   N   S   L   G   S   L   E   S   L   N   S   E   G   A   A   R   F   N

      910       920       930       940       950       960       970
GCC AAG TAC CCG CAG GGC ATC CCC ACC TCG GCC TGC GGC GAA GGC GCC TAC AAG GTC AAC GGC GTG AGC TAT TAC
A   K   Y   P   Q   G   I   P   T   S   A   C   G   E   G   A   Y   K   V   N   G   V   S   Y   Y

980       990       1000      1010      1020      1030      1040      1050
TCC TGG AGC GGT TCC TCG CCG CTG ACC AAC TTC CTC GAT CCG AGC GAC GCC TTC CTC GGC GCC TCG TCG CTG ACC
S   W   S   G   S   S   P   L   T   N   F   L   D   P   S   D   A   F   L   G   A   S   S   L   T

      1060      1070      1080      1090      1100      1110      1120      1
TTC AAG AAC GGC ACC GCC AAC GAC GGC CTG GTC GGC ACC TGC AGT TCG CAC CTG GGC ATG GTG ATC CGC GAC AAC
F   K   N   G   T   A   N   D   G   L   V   G   T   C   S   S   H   L   G   M   V   I   R   D   N

130       1140      1150      1160      1170      1180      1190      1200
TAC CGG ATG AAC CAC CTG GAC GAG GTG AAC CAG GTC TTC GGC CTC ACC AGC CTG TTC GAG ACC AGC CCG GTC AGC
Y   R   M   N   H   L   D   E   V   N   Q   V   F   G   L   T   S   L   F   E   T   S   P   V   S

      1210      1220      1230      1240      1250      1260      1270      1280      1
GTC TAC CGC CAG CAC GCC AAC CGC CTG AAG AAC GCC AGC CTG TAG GACCCCGGCCGGGGCCTCGGCCCCGGCCCTTTCCCGGAAG
V   Y   R   Q   H   A   N   R   L   K   N   A   S   L   *

290       1300      1310      1320      1330      1340      1350      1360      1370      1380      1
CCCCCTCGCGTGAAGAAAATCCTCCTGCTGATTCCACTGGCGTTCGCCGCCAGCCTGGCCTGGTTCGTCTGGCTGGAACCTTCCCCCCGCACCCGAGACGG

390       1400      1410      1420      1430      1440      1450      1460      1470 ORF2 1480
CGCCCCCGGCCAGCCCGCAGGCGGGCGCAGTCCACGCCCCGCCAGCAGCCTCCGCGGGAGAAGCGGTGCCGGCCCCTCAGGTC ATG CCG GCC AAG
                                                                                      M   P   A   K
```

```
     1490          1500          1510          1520          1530          1540          1550          1
GTC GCG CCG CTG CCA ACC TCC TTC AGG GGC ACC AGC GTC GAT GGC AGT TTC AGT GTC GAC GCC AGC GGC AAC CTG
 V   A   P   L   P   T   S   F   R   G   T   S   V   D   G   S   F   S   V   D   A   S   G   N   L

560           1570          1580          1590          1600          1610          1620          1630
CTG ATC ACC CGC GAC ATC CGC AAC CTG TTC GAC TAC TTC CTC AGC GCC GTC GGC GAA GAG CCC CTG CAG CAA AGC
 L   I   T   R   D   I   R   N   L   F   D   Y   F   L   S   A   V   G   E   E   P   L   Q   Q   S

     1640          1650          1660          1670          1680          1690          1700          1
CTG GAC CGC CTG CGC GCC TAC ATC GCC GCC GAA CTC CAG GAG CCG GCG CGC GGC CAG GCG TTG GCG CTG ATG CAG
 L   D   R   L   R   A   Y   I   A   A   E   L   Q   E   P   A   R   G   Q   A   L   A   L   M   Q

710           1720          1730          1740          1750          1760          1770          1780
CAA TAC ATC GAC TAC AAG AAG GAA CTG GTG CTG CTC GAA CGC GAC CTG CCG CGC CTG GCC GAC CTC GAC GCC CTG
 Q   Y   I   D   Y   K   K   E   L   V   L   L   E   R   D   L   P   R   L   A   D   L   D   A   L

     1790          1800          1810          1820          1830          1840          1850          1
CGC CAG CGG GAA GCC GCG GTG AAA GCC CTG CGC GCG CGG ATC TTC AGC AAC GAA GCG CAC GTG GCG TTC TTC GCC
 R   Q   R   E   A   A   V   K   A   L   R   A   R   I   F   S   N   E   A   H   V   A   F   F   A

860           1870          1880          1890          1900          1910          1920          1930
GAC GAG GAA ACC TAC AAC CAG TTC ACC CTG GAG CGC CTG GCG ATC CGC CAG GAC GGC AAG CTC AGC GCC GAG GAA
 D   E   E   T   Y   N   Q   F   T   L   E   R   L   A   I   R   Q   D   G   K   L   S   A   E   E

     1940          1950          1960          1970          1980          1990          2000          2
AAG GCC GCC GCC ATC GAC CGC CTG CGC GCC AGC CTG CCG GAA GAC CAG CAG GAA AGC GTG CTG CCG CAA CTG CAA
 K   A   A   A   I   D   R   L   R   A   S   L   P   E   D   Q   Q   E   S   V   L   P   Q   L   Q

010           2020          2030          2040          2050          2060          2070          2080
AGC GAA CTG CAG CAG CAG ACC GCC GCC CTC CAG GCC GCT GGC GCC GGC CCG GAA GCC ATC CGC CAG ATG CGT CAG
 S   E   L   Q   Q   Q   T   A   A   L   Q   A   A   G   A   G   P   E   A   I   R   Q   M   R   Q

     2090          2100          2110          2120          2130          2140          2150          2
CAA CTG GTG GGC GCC GAA GCC ACC ACC CGC CTG GAG CAA CTC GAT CGG CAA CGC TCG GCC TGG AAG GGC CGG CTG
 Q   L   V   G   A   E   A   T   T   R   L   E   Q   L   D   R   Q   R   S   A   W   K   G   R   L

160           2170          2180          2190          2200          2210          2220          2230
GAC GAC TAT TTC GCC GAG AAG AGC CGG ATC GAA GGC AAT ACC GGG GCT GAG CGA AGC CGA CCG CCG CGC GGC GGT
 D   D   Y   F   A   E   K   S   R   I   E   G   N   T   G   A   E   R   S   R   P   P   R   G   G

     2240          2250          2260          2270          2280          2290          2300          2
CGA AAC GCC TGG CCG AGG AGC GCT TCA GCG AAC AGG AAC GCT TGC GCC TGG GCG CGC TGG GAA CAG ATG CGC CAG
 R   N   A   W   P   R   S   A   S   A   N   R   N   A   C   A   W   A   R   W   E   Q   M   R   Q

310           2320          2330          2340          2350
GCC GAG CAG CGC TGA CCGGCACGGAAACGCCGAGAACGCGGCG
 A   E   Q   R   *
```

Fig. 2. Nucleotide sequence and deduced amino acid sequence of the lipase (ORF1, *lipA*; nucleotide positions 312–1247) and of an unknown protein (ORF2, *lipH*; nucleotide positions 1471–2322). Nucleotide sequence (upper line): ↓, start of pSW118; *, ribosome-binding sites; →←, inverted repeats (termination); --, restriction site for *SalI*. amino acid sequence (lower line, single-letter code): —, N-terminal amino acid sequence of the exported, mature lipase (Jaeger *et al.*, 1992); =, active centre of lipase.

verified by sequence analysis of the 3′ part of the construct and by the fact that lipase was expressed in *E. coli*.

Sequencing of lipA and properties of the lipase

A total of 2893 bp was sequenced in both directions, 2350 bp of which are shown in Fig. 2. This sequence covers the 0·5, 1·0 and 1·3 kb *SalI* fragments, and contains two open reading frames, ORF1 and ORF2. The G/C content of this DNA is 67 %. Codons ending with G or C are strongly preferred: 90 % for ORF1 and 86 % for ORF2. Both characteristics are typical for DNA of *P. aeruginosa* (West & Iglewski, 1988).

It was deduced that ORF1 is the lipase structural gene *lipA*. The open reading frame consists of 936 bp coding

Table 1. *Comparison of the amino acid sequence of the lipase from P. aeruginosa PAO1 with those of other Gram-negative bacteria*

Maximum homology analysis was done by applying PROSIS, considering identical and conserved amino acids as follows: A/S/T/P/G; N/D/E/Q; M/L/I/V; F/Y/W.

Strain compared	Maximum homology (%)	Amino acid sequences of active centres
Moraxella TA144 (*lip*3)	50	C G N S M G G
Moraxella TA144 (*lip*1)	51	I G W S M G G
P. cepacia	63	V G H S Q G G
P. glumae	63	I G H S Q G G
Pseudomonas sp. M-12-33	63	V G H S Q G G
P. fragi IFO-12049	64	I G H S Q G A
P. fragi IFO-3458	67	I G H S Q G A
Pseudomonas sp.*	89	V G H S H G G
Pseudomonas sp.†	99	I G H S H G G
Pseudomonas nov. sp. 109	100	I G H S H G G
	consensus:	- G - S - G -

* Cox *et al.*, 1991.
† Nishioka *et al.*, 1991.

Table 2. *Differences in the amino acid composition of lipases produced by three independently isolated pseudomonads*

Apart from the Q to H change all changes are equivalent.

Residue position	*P. aeruginosa* PAO1	*Pseudomonas* sp.*	*Pseudomonas* nov. sp. 109†
156	V	-----> I	
202	Q	-----> H	
204	I	-----> V	
125	I	---------->	M
126	A	---------->	P
176	T	---------->	A

* Nishioka *et al.*, 1991.
† Ihara *et al.*, 1991.

for a protein of 311 amino acids with M_r = 32721. Amino acid sequence analysis of the N-terminus of the purified lipase of *P. aeruginosa* PAC1R revealed the sequence of the exported, mature protein (Jaeger *et al.*, 1992). This sequence is identical to the deduced amino acid sequence of the *P. aeruginosa* PAO1 lipase, starting STYTQTKY . . . (underlined in Fig. 2). The sequence of the N-terminal 26 residues of the primary translation product is typical of leader sequences of secreted proteins (Watson, 1984). The mature protein deduced from the nucleotide sequence has a M_r of 30134. The isoelectric point was calculated, according to Sillero & Ribeiro (1989), to be 5·6. The polypeptide encoded by ORF1 includes an amino acid sequence known to be conserved in the active centre of lipases: I-G-H-S-H-G-G (Table 1). The overall amino acid composition corresponds well to that of known lipases, showing a high glycine (10·8 mol%), leucine (10·4 mol%) and serine (12·1 mol%) content. The amino acid sequence of the gene product was aligned with lipases from pseudomonads and *Moraxella*. High levels of homology were revealed especially to lipases from pseudomonads (>60%). There were only three variant residues when the sequence was aligned with lipases of *Pseudomonas* sp. (Nishioka *et al.*, 1991) and *Pseudomonas* nov. sp. 109 (Ihara *et al.*, 1991) (Table 2).

Hydropathy plots (Kyte & Doolittle, 1982) indicated several hydrophobic domains, the most striking of which are located between residues 33–57 and 152–171. The predicted active centre (residues 105–111) is embedded in a hydrophilic domain. A lipoprotein consensus sequence (LVGTCSS) exists at an unusual position near the C-terminus of the protein (residues 257–263).

A putative Shine–Dalgarno sequence (GAGA) was identified 9 bp 5′ to the ATG start codon of ORF1. No promoter sequence for genes of pseudomonads (Deretic *et al.* 1987) or *E. coli* could be found. Regulatory sequences corresponding to *crp*, *lexA*, *fur*, and *fnr* boxes were checked for upstream of the ORF1 start codon, but were not found.

Downstream of the TAG stop codon there are inverted repeats that can form hairpin structures in RNA for rho-independent termination of transcription.

ORF2 starts 220 bp downstream from ORF1 (Fig. 2). ORF2 (*lipH*) codes for a protein of 283 amino acids with an M_r of 33587. Hydropathy plots (Kyte & Doolittle, 1982) suggest it to be a soluble, cytoplasmic protein; it has extensive hydrophilic domains, especially at the C-terminus, and no signal sequence at the N-terminus. Homology of approximately 40 % to LimA of *P. cepacia* was found, with greater similarity in the C-terminal part of the protein (Fig. 3). LimA is coded by a sequence located directly downstream of the structural gene for the lipase of *P. cepacia* and is probably involved in the formation of an active extracellular enzyme (Jorgensen *et al.*, 1991; Nakanishi *et al.*, 1989).

Expression of the lipase

Plasmid pSW118 was transformed into *E. coli* JM109. Lipase activity was detectable only after induction with 1 mM-IPTG. Transcription of the gene was therefore under control of the *lac* promoter of pBluescript. After incubation at 37 °C for 16 h, no detectable lipase activity was found in cell-free culture medium. After disintegration of the cells by sonication, a lipase activity of 2·5 nmol min^{-1} ml^{-1} per 10^8 cells was measured (data corrected for esterase activity of *E. coli*).

Expression of the lipase gene in lipase-defective mutants 6-1 and 29-1 of *P. aeruginosa* PAO1 was tested using calcium triolein agar plates (Table 3). The broad-

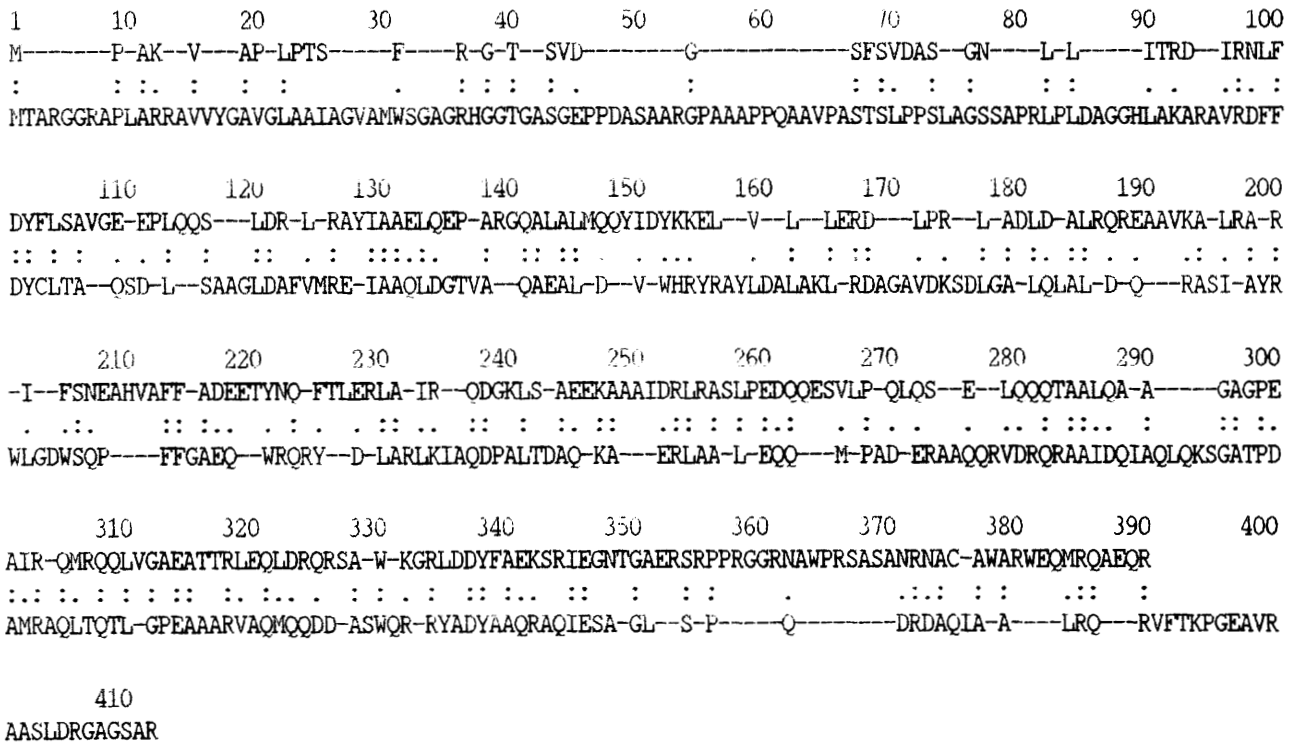

Fig. 3. Alignment of the amino acid sequences deduced from *lipH* of *P. aeruginosa* PAO1 and *limA* of *P. cepacia* (Jorgensen *et al.*, 1991). Upper line, LipH; lower line, LimA. Analysis was done with PROSIS. Symbols :, identical amino acids; ⊙, conserved amino acids (A/S/T/P/G; N/D/E/Q; M/L/I/V; F/Y/W).

host-range plasmid pSW118Ps carried the lipase gene (*lipA*) only, while plasmid pSW121Ps contained both ORF1 (*lipA*) and ORF2 (*lipH*) (Fig. 1). The lipase deficiency of mutant 29-1 could be complemented by introducing *lipA* alone, but expression of active extracellular lipase in mutant 6-1 was successful only in the presence of *lipH* (Kaudelka *et al.*, 1992).

Conservation of the lipase gene in pseudomonads

The two *Sal*I fragments of pSW101, including *lipH* and most of *lipA* of *P. aeruginosa* PAO1, were used as probes to study homology with lipase genes in other species of *Pseudomonas*. Under stringent conditions, all 10 strains of *P. aeruginosa* tested gave positive signals (Fig. 4). The *Sal*I restriction pattern has been mostly conserved, with the exception of the two *P. aeruginosa* strains CF_2/M_1 and Habs 12. They showed only a single band at about 2·2 kb. As this molecular mass equals the sum of the single fragments, no *Sal*I restriction site can be present within ORF2.

It is possible to distinguish between strains of *P. aeruginosa* and other lipase-producing pseudomonads by using this probe. *P. cepacia* DSM 50181, *P. putida* and *P. fluorescens* AFT 36 did not hybridize under either stringent or relaxed conditions. The latter experiments were performed with ^{32}P-radioactive as well as with digoxigenin-labelled probes. *P. maltophilia* DSM 50170 and *P. stutzeri* AS 70 gave weak but indistinct hybridization signals only under relaxed conditions. *P. alcaligenes* DSM 50342 was the only strain that showed a faint band under stringent conditions.

Based on these findings, we cloned the lipase gene of *P. alcaligenes* DSM 50342 on a 4·7 kb *Sal*I restriction fragment ligated into pUC19 (pCH1).

Table 3. *Complementation of lip mutants 6-1 and 29-1 of P. aeruginosa PAO1 with lipA and lipH*

Mutant strains were transformed with plasmid DNA. Lipase activity was detected on triolein plates after overnight incubation at 37 °C.

		Complementation of	
Plasmid	Gene(s)	6-1	29-1
pSW118Ps	*lipA*	−	+
pSW121Ps	*lipA* + *lipH*	+	+
pUC19Ps	−	−	−

+, Lipase activity.
−, No lipase activity.

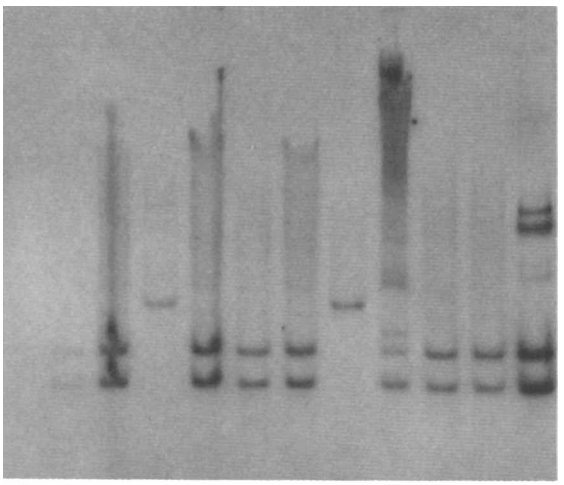

Fig. 4. Southern blot analysis of different strains of *P. aeruginosa*. Chromosomal DNA (10 μg) from each strain (1 μg DNA from *P. aeruginosa* PAO1) was digested with 30 U restriction enzyme *Sal*I. Southern hybridization was performed at 60 °C using ^{32}P-labelled double *Sal*I insert from plasmid pSW101 (1·0–1·3 kb). The insert codes for 85 % of the lipase gene (ORF1, *lipA*) and for the complete ORF2 (*lipH*). Lanes: 1, *E. coli* K12 (negative control); 2, *P. aeruginosa* PAO1; 3, *P. aeruginosa* CF_1/M_1; 4, *P. aeruginosa* CF_2/M_1; 5, *P. aeruginosa* CF_3/M_1; 6, *P. aeruginosa* DE-27; 7, *P. aeruginosa* Habs 2; 8, *P. aeruginosa* Habs 12; 9, *P. aeruginosa* ATCC 9027; 10, *P. aeruginosa* ATCC 27853; 11, *P. aeruginosa* FRD2; 12, pSW101 (positive control).

Discussion

Analysis of the lipase gene (lipA) and gene product (LipA)

The structural gene of the extracellular lipase (*lipA*) of *P. aeruginosa* PAO1 has been cloned, sequenced and expressed in both homologous and heterologous hosts.

Biochemical studies of purified lipase proteins of *P. aeruginosa* PAC1R (Stuer *et al.*, 1986; Jaeger *et al.*, 1991, 1992) and of *P. aeruginosa* EF2 (Gilbert *et al.*, 1991) yielded results which correspond very well to data deduced from the nucleotide sequence of the lipase gene of *P. aeruginosa* PAO1. These data comprise the molecular mass of the enzyme, the isoelectric point (calculated: pI = 5·6; analysed by isoelectric focussing: pI = 5·8), the overall amino acid composition and the amino acid sequence of the N-terminus of the secreted protein. The only exception was the high glycine content of the hydrolysate of the lipase.

The enzyme is probably synthesized as a propeptide of 311 residues, with a 26 residue leader peptide that is cleaved off during transport of the lipase across the cytoplasmic membrane. The signal sequence is composed of three positively charged amino acids at the N-terminus (KKK) followed by a stretch of hydrophobic amino acids. Besides this characteristic motif of a leader sequence, the amino acid sequence of the mature extracellular protein starts with STY.. (Fig. 2) just downstream of the putative signal protease cleavage site between A and S. SDS-PAGE of the purified extracellular lipase (Stuer *et al.*, 1986; Gilbert *et al.*, 1991) revealed an active extracellular enzyme of M_r 29000, corresponding well to the M_r of 30134 predicted for the mature protein from the nucleotide sequence. The entire lipase gene codes for a proenzyme of M_r 32721. Though no active lipase is found intracellularly (Stuer *et al.*, 1986), Koch and Jaeger immunologically detected an inactive protein of M_r 32000 (personal communication). These findings suggest that the extracellular lipase of *P. aeruginosa* PAO1 is produced as an inactive proenzyme with a leader peptide cleaved off during transport. Sequence data from other Gram-negative bacteria also reveal that these extracellular lipases are synthesized as proenzymes. In Gram-positive bacteria, extracellular lipases have to undergo a more complicated conversion involving a pre-proenzyme (Götz, 1991).

A special feature of the lipase protein of *P. aeruginosa* PAO1 is a stretch of 27 highly hydrophobic amino acids between residues 33 and 59 of the proenzyme at the N-terminus of the mature protein. This resembles a second leader sequence that is not cleaved off during export of the protein. This domain might be responsible for hydrophobic interactions between the enzyme and the lipopolysaccharides (LPS) released from the outer membrane. This hypothesis is based on findings of Stuer *et al.* (1986). They showed that the extracellular lipase of *P. aeruginosa* PAC1R is associated with LPS in cell-free growth medium and that the presence of LPS positively influences enzyme activity. As the lipase is an enzyme that mainly catalyses reactions at the water-lipid interphase it should have some hydrophobic domains for binding to such interphases. One such domain is found between residues 152 and 171.

A conserved sequence (IGHSHGG; Fig. 2) typical for many lipases and thought to be involved in lipolysis was found. No conclusions about amino acids belonging to the three-dimensional His-Ser-Gln/Asp triad can be drawn from primary structure alone.

Analysis of ORF2 (lipH) involved in expression of lipA

Sequence analysis revealed a second open reading frame (ORF2; *lipH*) downstream from *lipA*. It appears to encode a soluble cytoplasmic protein, in that it lacks a typical leader sequence. The function of LipH is yet unknown, but seems to be connected to lipase expression in *P. aeruginosa* PAO1. In complementation experiments (Table 3) we deduced that *lipH* codes for a protein that is required for the formation of an active extracellular

lipase. *lip* mutant 6-1, formally considered to be defective in *lipA* (Wohlfarth & Winkler, 1988) did not show lipase activity, either extracellularly or intracellularly. This mutant cannot be complemented by *lipA*. Recent sequencing results (Kaudelka *et al.*, 1992) indicated that mutant 6-1 is defective in *lipH* rather than in *lipA*. The gene product of *lipH* showed limited homology (40 %) to LimA, a protein that is presumed to be involved in lipase export in *P. cepacia* (Jorgensen *et al.*, 1991). Protein sequence comparisons did not show any similarity between LipH and XcpA (Bally *et al.*, 1991), XcpY or XcpZ (Filloux *et al.*, 1990), which are involved in protein export in *P. aeruginosa*. No motif common to activator proteins (e.g. LasR of two-component systems; Gambello & Iglewski, 1991) could be identified. We suggest that there is at least one other gene (*lipH*) specifically involved in the formation of an active extracellular lipase.

Expression of *lipA* in *E. coli* under control of the *lac* promoter is very low even after induction of enzyme synthesis by IPTG. Experiments should be done to verify whether the presence of *lipH* improves lipase expression in *E. coli*.

Active lipase is produced in very small amounts, even in the natural host strain (Stuer *et al.*, 1986). So far nothing is known about regulation of lipase expression and transport. Regulation by stress (SOS) or cAMP as was found for lipase expression in *Serratia marcescens* (Ball *et al.*, 1990; Winkler *et al.*, 1975) could not be confirmed for *P. aeruginosa* PAO1.

Conservation of lipA amongst pseudomonads

The structural genes for the lipases produced by various strains of *P. aeruginosa* showed a high degree of sequence homology by Southern hybridization analysis under stringent conditions. Moreover, Nishioka *et al.* (1991) and Ihara *et al.* (1991) published amino acid sequences deduced from lipase gene sequences of unidentified species of *Pseudomonas*. When comparing these sequences with that of the lipase studied here, it seemed very likely that both strains belong to the species *P. aeruginosa*. Both sequences differ by only three amino acids (Table 2). Actually, *Pseudomonas* sp. of Nishioka *et al.* (1991) was classified as *P. aeruginosa* TE3285 (Gilbert *et al.*, 1991). This very close overall nucleotide and amino acid sequence homology of the lipases of the three strains *P. aeruginosa* PAO1, *Pseudomonas* sp. (Nishioka *et al.*, 1991) and *Pseudomonas* nov. sp. 109 (Ihara *et al.*, 1991) as well as the identical N-termini of the mature lipase protein of *P. aeruginosa* PAC1R and EF2 (Jaeger *et al.*, 1992; Gilbert *et al.*, 1991) shows a considerable conservation of the lipase gene and gene-product amongst different strains of *P. aeruginosa*.

Sequence homologies between lipase genes are much less when comparing genes from distantly related pseudomonads. Data given in Table 1 and Southern blot analysis (Fig. 4) support this statement. *P. aeruginosa* PAO1, *P. alcaligenes* DSM 50342 and *Pseudomonas* sp. (P. M. Andreoli, personal communication) belong to rRNA group 1, and are thus very closely related. Their lipases also showed a high degree of homology at the amino acid (89 %) as well as the nucleotide level. More distantly related strains showed lower homology at the amino acid level (63% with *P. cepacia*) and less when comparing nucleotide sequences. In good agreement with these findings, Southern blot hybridizations under stringent conditions gave no positive signals. Vasil *et al.* (1986) obtained similar results when studying various strains of *P. aeruginosa* using the exotoxin A gene as a probe. Perhaps the lipase gene and lipase protein might be used to classify unknown species of *Pseudomonas*.

Description of gene copy

Palmeros *et al.* (1991) reported the cloning of a lipase gene from *P. aeruginosa* IGB83 that codes for a protein of M_r 54000. The activity of this enzyme was tested with tributyrine, a substrate that can be hydrolysed by lipases as well as unspecific esterases. It has to be verified that this enzyme is really a new type of lipase and is not a membrane-bound esterase (M_r 55000; Ohkawa *et al.*, 1979). Our Southern blot studies clearly showed that only a single copy of a lipase gene is present in the chromosome of *P. aeruginosa* PAO1 (data not shown). In *Moraxella* sp. TA144, however, Feller *et al.* (1991) discovered two different lipase genes (*lip1; lip3*) coding for proteins of nearly identical M_r (LipA, 34662; LipB, 34772).

We thank Drs S. Chen, B. Holloway, D. Ohman, and K. Timmis for kindly supplying us with bacterial strains and plasmids. We also acknowledge H. Kaudelka and B. Rehm who checked some parts of the nucleotide sequence. Part of this work was financially supported by Gist-Brocades, Delft (The Netherlands).

References

ANDREOLI, P. M., COX, M. M. J., FARIN, F. & WOHLFARTH, S. (1989). *Molecular cloning and expression of genes encoding lipolytic enzymes.* European Patent no. 0 334 462 A1.

AOYAMA, S., YOSHIDA, N. & INOUYE, S. (1988). Cloning, sequencing and expression of the lipase gene from *Pseudomonas fragi* IFO-12049 in *E. coli*. *FEBS Letters* **242**, 36–40.

BAGDASARIAN, M., LURZ, R., RÜCKERT, B., FRANKLIN, F. C. H., BAGDASARIAN, M. M., FREY, J. & TIMMIS, K. N. (1981). Specific-purpose plasmid cloning vectors. II. Broad host range, high copy number, RSF1010-derived vectors, and a host-vector system for gene cloning in *Pseudomonas*. *Gene* **16**, 237–247.

BALLY, M., BALL, G., BADERE, A. & LAZDUNSKI, A. (1991). Protein secretion in *Pseudomonas aeruginosa*: The *xcpA* gene encodes an integral inner membrane protein homologous to *Klebsiella pneumoniae* secretion function protein PulO. *Journal of Bacteriology* **173**, 479–486.

BRADY, L., BRZOZOWSKI, A. M., DEREWENDA, Z. S., DODSON, E., DODSON, G., TOLLEY, S., TURKENBURG, J. P., CHRISTIANSEN, L., HUGE-JENSEN, B., NORSKOV, L., THIM, L. & MENGE, U. (1990). A serine protease triad forms the catalytic centre of a triacylglycerol lipase. *Nature, London* **343**, 767–770.

CHEN, S. T., JORDAN, E. M., WILSON, R. B., DRAPER, R. K. & CLOWES, R. C. (1987). Transcription and expression of the exotoxin A gene of *Pseudomonas aeruginosa*. *Journal of General Microbiology* **133**, 3081–3091.

CHUNG, G. H., LEE, Y. P., YOO, O. J. & RHEE, J. S. (1991). Overexpression of a thermostable lipase gene from *Pseudomonas fluorescens* in *Escherichia coli*. *Applied Microbiology and Biotechnology* **35**, 237–241.

COX, M. M. J., ANDREOLI, P. M., GERRITSE, G. & QUAX, W. J. (1991). Molecular cloning, characterization and expression of a lipase gene from *Pseudomonas* species. In: *Book of Abstracts of Third International Symposium on Pseudomonad Biology and Biotechnology in Trieste*, p. 153.

DEININGER, P. L. (1983). Random subcloning of sonicated DNA: Application to shotgun DNA sequence analysis. *Analytical Biochemistry* **129**, 216–223.

DERETIC, V., GILL, J. F. & CHAKRABARTY, A. M. (1987). *Pseudomonas aeruginosa* infection in cystic fibrosis: nucleotide sequence and transcriptional regulation of the *algD* gene. *Nucleic Acids Research* **15**, 4567–4581.

FELLER, G., THIRY, M. & GERDAY, C. (1990). Sequence of a lipase gene from the antarctic psychrotroph *Moraxella* TA144. *Nucleic Acids Research* **18**, 6431.

FELLER, G., THIRY, M. & GERDAY, C. (1991). Nucleotide sequence of the lipase gene *lip3* from the antarctic psychrotroph *Moraxella* TA144. *Biochimica et Biophysica Acta* **1088**, 323–324.

FILLOUX, A., BALLY, M., BALL, G., AKRIM, M., TOMMASSEN, J. & LAZDUNSKI, A. (1990). Protein secretion in Gram-negative bacteria: transport across the outer membrane involves common mechanisms in different bacteria. *EMBO Journal* **13**, 4323–4329.

FRENKEN, L. G. J., BOS, J. W., VISSER, C. & VERRIPS, C. T. (1991). Isolation and characterization of the *Pseudomonas glumae* lipase gene and a second gene essential for lipase secretion. In: *Book of Abstracts of Third International Symposium on Pseudomonad Biology and Biotechnology in Trieste*, p. 153.

GAMBELLO, M. J. & IGLEWSKI, B. H. (1991). Cloning and characterization of the *Pseudomonas aeruginosa lasR* gene, transcriptional activator of elastase expression. *Journal of Bacteriology* **173**, 3000–3009.

GILBERT, E. J., CORNISH, A. & JONES, C. W. (1991). Purification and properties of extracellular lipase from *Pseudomonas aeruginosa* EF2. *Journal of General Microbiology* **137**, 2223–2229.

GÖTZ, F. (1991). Staphylococcal lipases and phospholipases. In *Lipases: Structure, Mechanism and Genetic Engineering* (GBF monograph no. 16), pp. 285–292. Edited by L. Alberghina, R. D. Schmid & R. Verger. Weinheim: VCH.

GÖTZ, F., POPP, F., KORN, E. & SCHLEIFER, K. H. (1985). Complete nucleotide sequence of the lipase gene from *Staphylococcus hyicus* cloned in *Staphylococcus carnosus*. *Nucleic Acids Research* **13**, 5895–5906.

GRAY, G. L., SMITH, D. H., BALDRIDGE, J. S., HARKINS, H. N., VASIL, M. L., CHEN, E. Y. & HEYNEKER, H. L. (1984). Cloning, nucleotide sequence, and expression in *Escherichia coli* of the exotoxin A structural gene of *Pseudomonas aeruginosa*. *Proceedings of the National Academy of Sciences of the United States of America* **81**, 2645–2649.

HENIKOFF, S. (1984). Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. *Gene* **28**, 351–359.

HOLLOWAY, B. W., KRISHNAPILLAI, V. & MORGAN, A. F. (1979). Chromosomal genetics of *Pseudomonas*. *Microbiological Reviews* **43**, 73–102.

IHARA, F., KAGEYAMA, Y., HIRATA, M., NIHIRA, T. & YAMADA, Y. (1991). Purification, characterization, and molecular cloning of lactonizing lipase from *Pseudomonas* species. *Journal of Biological Chemistry* **266**, 18135–18140.

JAEGER, K.-E., WOHLFARTH, S. & WINKLER, U. K. (1991). Extracellular lipase of *Pseudomonas aeruginosa*. In *Lipases: Structure, Mechanism and Genetic Engineering* (GBF monograph no. 16), pp. 381–384. Edited by L. Alberghina, R. D. Schmid & R. Verger. Weinheim: VCH.

JAEGER, K.-E., ADRIAN, F.-J., MEYER, H. E., HANCOCK, R. E. W. & WINKLER, U. K. (1992). Extracellular lipase from *Pseudomonas aeruginosa* is an amphiphilic protein. *Biochima et Biophysica Acta*, in the Press.

JORGENSEN, S., SKOV, K. W. & DIDERICHSEN, B. (1991). Cloning, sequence, and expression of a lipase gene from *Pseudomonas cepacia*: Lipase production in heterologous hosts requires two *Pseudomonas* genes. *Journal of Bacteriology* **173**, 559–567.

KAUDELKA, H., WOHLFARTH, S. & WINKLER, U. (1992). Molecular characterization of two lipase-defective mutants of *Pseudomonas aeruginosa*. *BioEngineering* **8** (*Abstracts of Gemeinsame Frühjahrstagung der VAAM und DHGM*), 79.

KUGIMIYA, W., OTANI, Y., HASHIMOTO, Y. & TAKAGI, Y. (1986). Molecular cloning and nucleotide sequence of the lipase gene from *Pseudomonas fragi*. *Biochemical and Biophysical Research Communications* **141**, 185–190.

KYTE, J. & DOOLITTLE, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *Journal of Molecular Biology* **157**, 105–132.

LEE, C. Y. & IANDOLO, J. J. (1986). Lysogenic conversion of staphylococcal lipase is caused by insertion of the bacteriophage L54a genome into the lipase structural gene. *Journal of Bacteriology* **166**, 385–391.

MARMUR, J. (1961). A procedure for the isolation of deoxyribonucleic acid from micro-organisms. *Journal of Molecular Biology* **3**, 208–218.

NAKANISHI, Y., KURONO, Y., KOIDE, Y. & BEPPU, T. (1989). Recombinant DNA, bacterium of the genus pseudomonas containing it, and process for preparing lipase by using it. *European Patent* No. 0 331 376 A2.

NAKANISHI, Y., WATANABE, H., WASHIZU, K., NARAHASHI, Y. & KURONO, Y. (1991). Cloning, sequencing and regulation of the lipase gene from *Pseudomonas* sp. M-12-33. In *Lipases: Structure, Mechanism and Genetic Engineering* (GBF monograph no. 16), pp. 263–266. Edited by L. Alberghina, R. D. Schmid & R. Verger. Weinheim: VCH.

NISHIOKA, T., CHIHARA-SHIOMI, M., YOSHIKAWA, K., INAGAKI, M., YAMAMOTO, Y., HIRATAKE, J., BABA, N. & ODA, J. (1991). Lipase from *Pseudomonas* sp.: Reactions, cloning, and amino acid sequence analysis. In *Lipases: Structure, Mechanism and Genetic Engineering* (GBF monograph no. 16), pp. 253–262. Edited by L. Alberghina, R. D. Schmid & R. Verger. Weinheim: VCH.

OHKAWA, I., SHIGA, S. & KAGEYAMA, M. (1979). An esterase on the outer membrane of *Pseudomonas aeruginosa* for the hydrolysis of long chain acyl esters. *Journal of Biochemistry* **86**, 643–656.

O'HOY, K. & KRISHNAPILLAI, V. (1987). Recalibration of the *Pseudomonas aeruginosa* PAO chromosome map in time units using high-frequency-of-recombination donors. *Genetics* **115**, 611–618.

PALMEROS, B., TAMAYO, E. M., RAMIREZ, G., NAJERA, R. & SOBERON-CHAVES, G. (1991). Cloning and characterization of a *Pseudomonas aeruginosa* lipase. In: *Book of Abstracts of Third International Symposium on Pseudomonad Biology and Biotechnology in Trieste*, p. 167.

SAMBROOK, J., FRITSCH, E. F. & MANIATIS, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd Edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

SANGER, F., NICKLEN, S. & COULSON, A. R. (1977). DNA sequencing with chain terminating inhibitors. *Proceedings of the National Academy of Sciences of the United States of America* **74**, 5463–5467.

SCHRAG, J. D., LI, Y., WU, S. & CYGLER, M. (1991). Ser-His-Glu triad forms the catalytic site of the lipase from *Geotrichum candidum*. *Nature, London* **351**, 761–764.

SILLERO, A. & RIBEIRO, J. M. (1989). Isoelectric points of proteins: Theoretical determination. *Analytical Biochemistry* **179**, 319–325.

STADEN, R. (1980). A new computer method for the storage and manipulation of DNA gel reading data. *Nucleic Acids Research* **8**, 3673–3694.

STUER, W., JAEGER, K. E. & WINKLER, U. K. (1986). Purification of extracellular lipase from *Pseudomonas aeruginosa*. *Journal of Bacteriology* **168**, 1070–1074.

VASIL, M. L., CHAMBERLAIN, C. & GRANT, C. C. R. (1986). Molecular studies of *Pseudomonas* exotoxin A gene. *Infection and Immunity* **52**, 538–548.

WATSON, M. E. E. (1984). Compilation of published signal sequences. *Nucleic Acids Research* **13**, 5145–5164.

WEST, S. E. H. & IGLEWSKI, B. H. (1988). Codon usage in *Pseudomonas aeruginosa*. *Nucleic Acids Research* **16**, 9323–9335.

WINKLER, U., SCHOLLE, H. & BOHNE, L. (1975). Mutants of *Serratia marcescens* lacking cyclic nucleotide phosphodiesterase activity and requiring cyclic 3′,5′-AMP for the utilization of various carbohydrates. *Archives of Microbiology* **104**, 189–196.

WINKLER, F. K., D'ARCY, A. & HUNZIKER, W. (1990). Structure of human pancreatic lipase. *Nature, London* **343**, 771–774.

WOHLFARTH, S. & WINKLER, U. K. (1988). Chromosomal mapping and cloning of the lipase gene of *Pseudomonas aeruginosa*. *Journal of General Microbiology* **134**, 433–440.

YANISCH-PERRON, C., VIEIRA, J. & MESSING, J. (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* **33**, 103–119.