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

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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 preenzyme of 311 amino acids including a leader sequence of 26 amino acids. The mature protein was predicted to have a *M* 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*, 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.


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* of 29000 and the mature proteins have the same N-terminal sequences (STYQT-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. (Nishioaka *et al.*, 1991) and from *Pseudomonas* nov. sp. 109 (Ihara *et al.*, 1991) also revealed an apparent *M* 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
57 min (old map) or 39 min (map according to O'Hoy & Krishnapillai, 1987) and it has been cloned on a 3-1 kb SalI 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 CF2/M1, CF4/M1, CF5/M1 (all from the stock culture collection of the institute, Bochum, FRG). *P. fluorescens* ATF36, *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 paaA1 lip1) and 6-1 (met-9020 catA1 paaA1 lip2). *E. coli* JM109 (recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 lacI5175 [lac-proAB] (F' traD36 proAB lacI ZAM15) 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 pRC254 (Chen et al., 1987) inserted into PstI 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 Im4·IPTG. To grow *E. coli* for transformation, 20 mM-MgCl₂ (equal amounts of MgSO₄ and MgCl₂) 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 CaCl₂ method (Sambrook et al., 1989). After growing *E. coli* in the presence of 20 mM-MgCl₂ washed cells were suspended in 40 mM-MnCl₂, 100 mM-CaCl₂ and 50 mM-RbCl. For transformation of *P. aeruginosa*, early exponential growth phase cells were washed twice with 150 mM-MgCl₂. 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 32P-dATP (0.37 MBq, Amersham) and the Klaveno fragment of DNA polymerase. To prevent sequence compressions, 7-deaza-dGTP was used instead of dGTP. 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 32P-dATP or using fluorescently labelled universal or reversed primers 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 32P-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-Labeling 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 SalI 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 PA01 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 Sall 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: BamHI; E, EcoRI; S, SalI; X, XhoI. Genes lipA and lipH are included as boxes; arrows within indicate direction of transcription.

Two Sall fragments (1.0–1.3 kb) were inserted into the Sall 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 Sall fragment, which carries 85% of the gene, was subsequently used as a probe to clone the intact gene.

Genomic DNA from P. aeruginosa PA01 was digested with restriction endonuclease PvuII, for which there is no cleavage site within the Zip 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 Sall 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 Sall fragments, including the 1.0 kb and 1.3 kb fragments present in pSW101 (verified by Southern hybridization, data not shown).

Restriction enzyme EcoRI, which cuts within the 1.0 kb Sall 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 EcoRI/BamHI fragment into pUC19 cleaved with the same enzymes. The resulting plasmid pSW117-2 was further reduced in size by subcloning an EcoRI/XhoI fragment. The ends of this fragment were made blunt before ligation into Sall site of pUC19 to form plasmid pSW117-3. The insert of pSW117-3 was subjected to progressive exonuclease digestion (Henikoff, 1984) starting from the BamHI restriction site within the vector directly adjacent to the filled-in XhoI 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 Sall/EcoRI fragment of plasmid pSW117-8 (the Sall site comes from the lipase gene and the EcoRI 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 Sall fragment into the Sall 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
Fig. 2. Nucleotide sequence and deduced amino acid sequence of the lipase (ORF1, lipA; nucleotide positions 312-1247) and of an unknown protein (ORF2, nP; nucleotide positions 1471-2322). Nucleotide sequence (upper line): 1, start of pSW18; *, ribosome-binding sites; +, inverted repeats (termination); --, restriction site for Sall. 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.

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 Sall 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 verified by sequence analysis of the 3' part of the construct and by the fact that lipase was expressed in E. coli.
Table 1. Comparison of the amino acid sequence of the lipase from P. aeruginosa PAOl 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.

<table>
<thead>
<tr>
<th>Strain compared</th>
<th>Maximum homology (%)</th>
<th>Amino acid sequences of active centres</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moraxella TA144 (lip3)</td>
<td>50</td>
<td>C G N S M G G</td>
</tr>
<tr>
<td>Moraxella TA144 (lip1)</td>
<td>51</td>
<td>I G W S M G G</td>
</tr>
<tr>
<td>P. cepacia</td>
<td>63</td>
<td>V G H S Q G G</td>
</tr>
<tr>
<td>P. glumae</td>
<td>63</td>
<td>I G H S Q G G</td>
</tr>
<tr>
<td>Pseudomonas sp. M-12-33</td>
<td>63</td>
<td>V G H S Q G G</td>
</tr>
<tr>
<td>P. fragi IFO-12049</td>
<td>64</td>
<td>I G H S Q G A</td>
</tr>
<tr>
<td>P. fragi IFO-3458</td>
<td>67</td>
<td>I G H S Q G A</td>
</tr>
<tr>
<td>Pseudomonas sp.</td>
<td>89</td>
<td>V G H S H G G</td>
</tr>
<tr>
<td>Pseudomonas sp.†</td>
<td>99</td>
<td>I G H S H G G</td>
</tr>
<tr>
<td>Pseudomonas nov. sp. 109</td>
<td>100</td>
<td>I G H S H G G</td>
</tr>
</tbody>
</table>

* 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.

<table>
<thead>
<tr>
<th>Residue position</th>
<th>P. aeruginosa</th>
<th>Pseudomonas sp.</th>
<th>Pseudomonas nov. sp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>156</td>
<td>V</td>
<td>----- &gt; I</td>
<td>109†</td>
</tr>
<tr>
<td>202</td>
<td>Q</td>
<td>----- &gt; H</td>
<td></td>
</tr>
<tr>
<td>204</td>
<td>I</td>
<td>----- &gt; V</td>
<td></td>
</tr>
<tr>
<td>125</td>
<td>I</td>
<td>--------------- &gt; M</td>
<td></td>
</tr>
<tr>
<td>126</td>
<td>A</td>
<td>--------------- &gt; P</td>
<td></td>
</tr>
<tr>
<td>176</td>
<td>T</td>
<td>--------------- &gt; A</td>
<td></td>
</tr>
</tbody>
</table>

† 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 PAOl 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 30 134. The isoelectric point was calculated, according to Siller & 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 33 587. 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 10⁸ 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 PAOl was tested using calcium triolein agar plates (Table 3). The broad-
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 SalI fragments of pSW101, including lipH and most of lipA of P. aeruginosa PA01, 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 SalI restriction pattern has been mostly conserved, with the exception of the two P. aeruginosa strains CF2/M1 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 SalI 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 ³²P-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 SalI restriction fragment ligated into pUC19 (pCH1).

Table 3. Complementation of lip mutants 6-1 and 29-1 of P. aeruginosa PA01 with lipA and lipH

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Gene(s)</th>
<th>6-1</th>
<th>29-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSW118Ps</td>
<td>lipA</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>pSW121Ps</td>
<td>lipA + lipH</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pUC19Ps</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

+ Lipase activity.
- No lipase activity.
Discussion

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

The structural gene of the extracellular lipase (lipA) of P. aeruginosa PA01 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 PA01. These data comprise the 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, 29000, corresponding well to the M, of 30134 predicted for the mature protein from the nucleotide sequence. The entire lipase gene codes for a proenzyme of M, 32721. Though no active lipase is found intracellularly (Stuer et al., 1986), Koch and Jaeger immunologically detected an inactive protein of M, 32000 (personal communication). These findings suggest that the extracellular lipase of P. aeruginosa PA01 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 PA01 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 PA01. 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 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. alcgalgenes 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, 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, 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, (LipA, 34662; LipB, 34772).

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References


