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Degradation of Triglycerides by a Pseudomonad Isolated from Milk: Molecular Analysis of a Lipase-Encoding Gene and Its Expression in *Escherichia coli*

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Psychrotrophic lipolytic bacteria represent a significant problem in the storage of refrigerated dairy products. A lipase-encoding gene has been cloned and characterized from a highly lipolytic strain of *Pseudomonas*. The nucleotide sequence of the gene predicts a polypeptide of M_r 49,905, which was identified when the gene was expressed in *Escherichia coli*.

Psychrotrophic bacteria represent a significant problem in the storage of refrigerated milk and dairy products because of their production of extracellular hydrolytic enzymes (7, 8, 10). Spoilage due to the action of lipases occurs predominantly because of the production of short- to medium-chain (C_4 to C_{12}), even-carbon-number fatty acids from milk triglycerides (8). Pseudomonads, particularly *Pseudomonas fluorescens* and *Pseudomonas fragi*, are the predominant lipolytic psychrotrophs in raw and ultra-heat-treated milk (9, 20, 21). Lipases from a number of pseudomonads are very heat stable, and several have been purified (1, 2, 13, 18, 19, 27).

We have initiated an investigation of the genetic and biochemical bases of the lipolytic phenotype of a psychrotrophic pseudomonad (LS107d2) which is taxonomically similar to *P. fluorescens* and was isolated from caprine milk (9). A pertinent characteristic of strains potentially able to cause lipolytic spoilage of milk is their ability to degrade triglycerides in butterfat, resulting in zones of clearing in solid media containing an emulsion of 0.1% butterfat (22). By this criterion, LS107d2 is markedly lipolytic, and more so than other strains of *Pseudomonas* tested (Fig. 1). Very similar results were obtained with media containing an emulsion of 0.1% glyceryl trioleate (TO; data not shown). We describe the isolation and characterization of a lipase-encoding gene from this strain.

Isolation of a lipase-encoding gene (*lipA*) from *Pseudomonas* strain LS107d2. A genomic library was constructed in the cosmid vector pHC79 (12). Individual recombinants were patched onto media containing TO and incubated at 30°C for 5 to 10 days. One recombinant (pTO1) of 3,000 screened gave a zone of clearing and was chosen for further study. Cosmid DNA was isolated, DNA fragments generated by partial *Taq*I digestion were ligated with *Acc*I-cleaved pUC118 (26), and the resulting transformants were screened for lipase activity on TO-containing media. One transform-

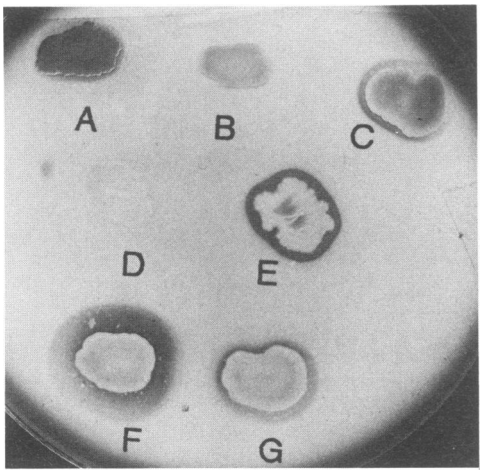

FIG. 1. Lipolytic phenotypes of *Pseudomonas* and *E. coli* strains on medium containing butterfat. Incubation was at 30°C for 4 days. (A) *P. aeruginosa* PAO 2302; (B) *P. fluorescens* ATCC 13525; (C) *P. fragi* ATCC 4973; (D) *E. coli* TG1; (E) *E. coli* TG1 containing pLAJ2; (F) *Pseudomonas* strain LS107d2; (G) *Pseudomonas* strain I8 (*P. fragi* from caprine milk).

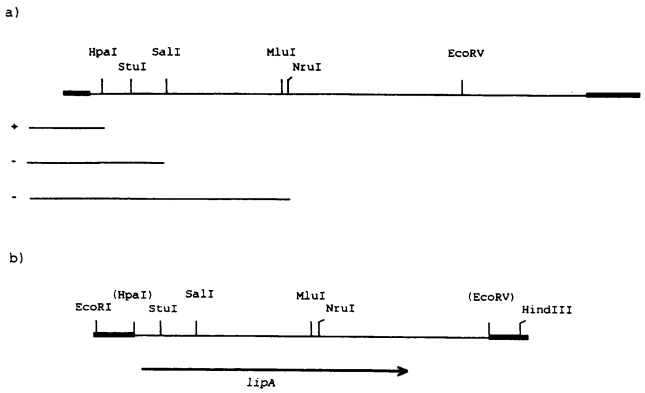

FIG. 2. Restriction map of the *lipA* coding region of *Pseudomonas* strain LS107d2. Dark lines represent vector sequences, and thin lines represent genomic DNAs. (a) Restriction map of the DNA insert in pLGO11. The lipolytic phenotypes of deletion derivatives are indicated beneath the map. Each deletion extends from a corresponding restriction endonuclease site within the vector. (b) Restriction map of pLAJ2. The hybrid *Sma*I-*Hpa*I and *Sma*I-*Eco*RV sites are shown in parentheses. Only relevant restriction enzyme sites in the vector polylinker sequence are shown. The *lipA* coding sequence is indicated beneath the map.

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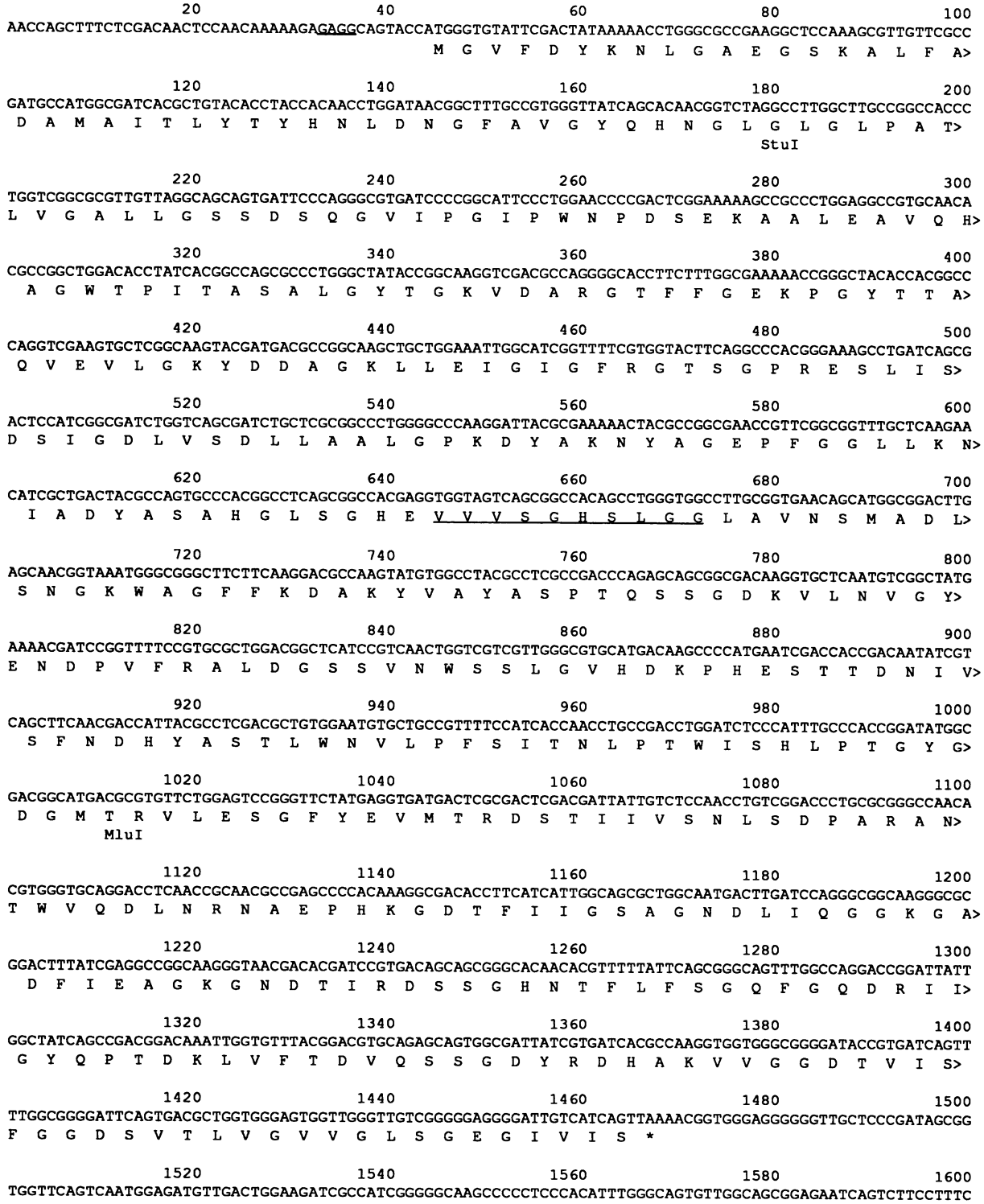

FIG. 3. Nucleotide sequence of the *lipA* region. Nucleotide sequencing was performed by the dideoxy chain termination method with T7 DNA polymerase (25). DNA fragments were either cloned into pUC118 and sequenced as supercoiled templates (15) or cloned into M13mp18 or M13mp19 with single-stranded DNA as template (17). Numbers above the sequence denote nucleotide positions. The deduced amino acid sequence of the *lipA* gene product is given below the nucleotide sequence. The Shine-Dalgarno sequence and the putative active-site region of the lipase are underlined.

ant, which was lipolytic, contained a deletion derivative of pTO1, designated pLGO11, with a 2.0-kb insert (Fig. 2a). Deletions derived from pLGO11 (Fig. 2a) localized the gene which encodes the lipase (*lipA*) to the right of the *Hpa*I site. An *Hpa*I-*Eco*RV fragment was subcloned into the *Sma*I site of pUC118 to give pLAJ2 (Fig. 2b); *Escherichia coli* containing pLAJ2 displayed a lipolytic phenotype on both butterfat (Fig. 1) and TO-containing media (data not shown). Cells containing a recombinant plasmid in which the *Hpa*I-*Eco*RV fragment is cloned in the opposite orientation are Lip$^-$, indicating that expression involves a vector promoter.

The nucleotide sequence of *lipA*, and the *lipA*-encoded polypeptide. The nucleotide sequence of the DNA fragment in pLAJ2 revealed an open reading frame of 1,412 bp (Fig. 3) encoding a polypeptide with a calculated mass of 49,905. The *lipA* region, derived as a *Hin*dIII-*Eco*RI fragment from pLAJ2, was cloned into pT7-5 to yield pLJ1. The molecular mass of the *lipA*-encoded polypeptide expressed in *E. coli* containing this plasmid was determined by selective labeling with [^{35}S]methionine followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (24). A single polypeptide with a molecular mass of 50,000 was evident (data not shown); this was in good agreement with the calculated molecular mass of 49,905.

In order to correlate the open reading frame with *lipA*, deletions were created at the *Mlu*I and *Stu*I sites (Fig. 2). Following cleavage with *Stu*I or *Mlu*I, pLJ1 was treated with *Bal* 31 nuclease or mung bean nuclease to create a deletion of 161 or 7 bp, respectively (nucleotides 102 to 262 and 1009 to 1015, respectively; Fig. 3). *E. coli* containing pLJ1 displayed a Lip$^+$ phenotype on media containing glyceryl tributyrate or TO, presumably because of expression from a fortuitous vector promoter. However, cells containing the deleted derivatives of pLJ1 were Lip$^-$, indicating that these deletions are located within the *lipA* open reading frame. Furthermore, labeling with [^{35}S]methionine revealed that the *lipA* polypeptide was not expressed (data not shown).

The initiating methionine codon shown in Fig. 3 is strongly indicated by the presence of a Shine-Dalgarno sequence (11) 7 bp upstream. A possible alternative is an in-frame AUG codon 21 codons downstream (nucleotides 107 to 109; Fig. 3), although a corresponding Shine-Dalgarno sequence is not evident. To confirm the identity of the initiating AUG codon, advantage was taken of the inclusion of both AUG codons within *Nco*I restriction sites. An *Nco*I-*Nco*I deletion was therefore created by using pLJ1, essentially placing the second methionine codon at the former position of the upstream methionine codon and deleting the intervening 62 bp. When this deleted *lipA* gene is expressed and labeled with [^{35}S]methionine by using the T7 polymerase system (24), the gene product is, as expected, detectably smaller in molecular mass than the undeleted *lipA* gene product (data not shown). However, *E. coli* cells containing this deletion derivative of pLJ1 are phenotypically Lip$^-$, indicating that the upstream AUG codon is correctly identified and also that the N-terminal 21 amino acids are essential for lipase activity.

The usage of synonomous codons in *lipA* reveals a marked bias (data not shown), which is quite similar to that observed for *Pseudomonas aeruginosa* (28). In particular, the third codon position is commonly a G or a C, which is consistent with the high mole percent G+C contents of the genomes of *Pseudomonas* species.

The *lipA* gene product is clearly a true lipase, since it is able to hydrolyze insoluble substrates (6, 23), namely, TO and *p*-nitrophenylpalmitate (Fig. 1; 16). The amino acid

Staphylococcus aureus	V	H	L	V	G	H	S	M	G	G
Staphylococcus hyicus	V	H	F	I	G	H	S	M	G	G
Pseudomonas cepacia	V	N	L	V	G	H	S	Q	G	G
Pseudomonas fluorescens	V	V	V	S	G	H	S	L	G	G
Pseudomonas fragi	V	N	L	I	G	H	S	Q	G	A
Rhizomucor miehei	V	A	V	T	G	H	S	L	G	G
Porcine pancreatic lipase	V	H	V	I	G	H	S	L	G	S
Rat lingual lipase	I	H	Y	V	G	H	S	Q	G	T
Rat hepatic lipase	V	H	L	I	G	Y	S	L	G	A
Mouse lipoprotein lipase	V	H	L	L	G	Y	S	L	G	A

FIG. 4. Alignment of putative active-site regions of microbial and mammalian lipases. Highly conserved residues are boxed. The *P. fluorescens* sequence is *lipA* (Fig. 3). The other sequences may be found in references 3 and 14.

sequence of the *lipA*-encoded lipase deduced from the nucleotide sequence shows no extensive similarity to the sequence of lipases from *Pseudomonas cepacia* (14) or *P. fragi* (12). A putative active-site serine residue, however, is located within a sequence (Fig. 3) which is conserved in bacterial and mammalian lipases (3, 5); these sequences are aligned in Fig. 4.

Localization of lipase in *E. coli*. The lipases so far described in *Pseudomonas* species are exported to the medium (2, 13, 18, 19, 27), which is consistent with the large molecular size of insoluble triglyceride substrates relative to the permeability of the outer membrane (4). Lipase activity from *Pseudomonas* strain LS107d2 is also found to be extracellular (16). It was therefore of interest to determine its cellular localization when it is expressed in *E. coli*.

E. coli containing pLAJ2 was fractionated to yield periplasmic and cytoplasm-plus-membrane fractions. Lipase activity was consistently and exclusively found in the periplasmic fraction, whereas the majority of the marker enzymes β-lactamase and β-galactosidase was located in the periplasm and the cytoplasm-plus-membrane fractions as expected (data not shown). No activity was detected in the medium. When cells containing the *lipA*-encoded polypeptide labeled with [^{35}S]methionine were similarly fractionated, a significant proportion of the polypeptide was located in the cytoplasm (data not shown). In view of the lack of activity in the cytoplasm, this intracytoplasmic lipase is presumably inactive. The mechanism by which at least a portion of the lipase is secreted to the periplasm remains to be determined. The export of lipase to the culture fluid in *Pseudomonas* strain LS107d2 likewise merits investigation but seems to involve other gene products in view of the absence of such export in *E. coli*.

The identification of *lipA* will allow detailed studies on the role of this lipase in the hydrolysis of butterfat triglycerides and in lipolytic spoilage.

Nucleotide sequence accession number. The nucleotide sequence reported in Fig. 3 has been submitted to GenBank and has the accession number M7412.

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