Molecular analysis of an esterase-encoding gene from a lipolytic psychrotrophic pseudomonad

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An esterase gene (*estA*) from a lipolytic psychrotroph (*Pseudomonas* sp. LS107d2), was cloned in *Escherichia coli* and its nucleotide sequence was determined, revealing an ORF encoding a polypeptide of 389 amino acid residues, with a molecular mass of 42276 Da. Labelling of plasmid-encoded proteins with [³⁵S]methionine, using the maxicell procedure, gave a single polypeptide of molecular mass 42 kDa, consistent with that calculated from the ORF. Colonies of *E. coli* cells containing *estA* produced a clear halo when grown on solid media containing tributyrin; no clearance was produced when cells were grown on media containing triolein. Extracts of cells containing *estA* also hydrolysed water-soluble nitrophenol esters, but were unable to cleave water-insoluble substrates. The preference for water-soluble substrates indicates that the gene product is an esterase.

Introduction

The significance of psychrotrophic organisms in the spoilage of milk is widely recognized (Cousin, 1989). Milk spoilage can occur because of the accumulation of free fatty acids produced from the hydrolysis of triacylglycerides by lipases. Organisms of the genus *Pseudomonas* constitute the most common psychrotroph isolated from milk (Muir *et al.*, 1979; Cousin, 1982; Deeth & Fitzgerald, 1983). In particular, psychrotrophic pseudomonads dominate the lipolytic microflora of raw bovine milk samples which show lipolytic defects, and caprine milk samples which have been refrigerated for several days (Shelley *et al.*, 1986; Cox & MacRae, 1989). *Pseudomonas fluorescens* and *Pseudomonas fragi* have been identified as the most predominant lipolytic psychrotrophic species; the relative importance of these two species in lipolytic spoilage may depend on other factors such as initial cell numbers, growth rates and, in

regard to products derived from heat-treated milk, the thermostability of relevant enzymes (Griffiths, 1981; Shelley *et al.*, 1986; Cox & MacRae, 1989). Lipolytic spoilage is mainly due to the production of even-carbon-number short-to-medium chain (C4 to C12) fatty acids from triacylglycerides (Cousin, 1989). Because nearly all triacylglycerides are completely insoluble in water, the enzymes responsible for initial hydrolysis are, by definition, lipases since they act at an oil–water interface. It is unclear, however, whether esterases, which act only on soluble substrates, also play a part in lipolytic spoilage by hydrolysing soluble mono- and diacylglycerides. As part of a study on the individual enzymes involved in lipolytic spoilage, and the genes which encode them, we have initiated a molecular genetic analysis of strains of psychrotrophic lipolytic pseudomonads isolated from caprine milk. We describe the isolation and characterization of a gene which encodes an esterase active on tributyrin, and on artificial substrates (nitrophenol esters) with acyl chain lengths up to C10:0.

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Abbreviations: BFA, butter fat agar; TBA, tributyrin agar; TOA, triolein agar.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M68491.

Methods

Bacterial strains, plasmids and media. The *Pseudomonas* strain used in this study, LS107d2, was one of a number of strains isolated in a survey of proteolytic and lipolytic psychrotrophs in caprine milk (Cox & MacRae, 1989). It derives from phenon G of this study, and resembles

Pseudomonas fluorescens. *E. coli* strain LE392 (*supE44 supF58 hsdR514 galK2 galT22 metB1 trpR55 lacY1*; Sambrook *et al.*, 1989) was the host strain used for cosmid bank construction and TG1 (*supE hsdΔ5 thiΔ(lac-proAB) F'[traD36 proAB⁺ lacI^s lacZΔM15]*; Sambrook *et al.*, 1989) was used for all other genetic manipulations and for enzyme assays. The cosmid vector pHC79 (Hohn & Collins, 1980) was used for cosmid library constructions. pHP34 (Prentki & Krisch, 1982) and pSK (BLUESCRIPT M13+; Stratagene), were employed as cloning vectors. For general strain maintenance and recombinant DNA manipulation, LB medium (Sambrook *et al.*, 1989) was used, either as broth, or solidified with 1.5% (w/v) bacteriological agar. Butterfat agar (BFA), containing 0.1% butterfat, was made according to Shelley *et al.* (1987). Tributyrin agar (TBA) and triolein agar (TOA) were made in the same way as BFA, except that 1% (v/v) tributyrin or 0.1% triolein were added in place of butterfat. Where appropriate, ampicillin and tetracycline were added to final concentrations of 50 µg ml⁻¹ and 20 µg ml⁻¹, respectively.

Cosmid library construction and screening. Chromosomal DNA was isolated from LS107d2 and purified on a caesium chloride gradient as described by Sambrook *et al.* (1989). The DNA was partially digested with *Sau3A* to yield high-molecular-mass fragments, and then ligated with *Bam*HI-digested pHC79 without prior fractionation. The ligation mix was packaged, using 'Packagene' extract (Promega), transfected into LE392, and plated onto LB plates containing ampicillin. To screen for hydrolytic activity, individual recombinants were patched onto BFA or TBA plates and incubated at 30 °C for 5–10 d; hydrolytic activity is indicated by zones of clearing around the colonies.

Recombinant DNA techniques. Unless described in detail, all recombinant DNA methods were essentially as described by Sambrook *et al.* (1989).

Creating overlapping unidirectional deletions using exonuclease III and mung bean nuclease. The method was essentially as described by Henikoff (1984). The fragment to be analysed was cloned into pSK and plasmid DNA was prepared. The plasmid DNA was digested with two enzymes, one leaving a 3' single stranded overhang and the other a blunt end or 5' overhang. The digested DNA was then incubated at 37 °C in a reaction mixture containing (per time point) 1 µg DNA, 2.5 µl 2 × exonuclease III buffer (100 mM-Tris/HCl, pH 8.0, 10 mM-MgCl₂, 20 µg ml⁻¹ tRNA), 0.5 µl β-mercaptoethanol, 0.2 µl exonuclease III (Promega, 25 units µl⁻¹), in a final volume of 5 µl. Reaction mixture (5 µl) was removed at 60 s intervals to tubes containing 4 µl of 10 × buffer (300 mM-NaOAc, pH 5.0, 500 mM-NaCl, 10 mM-ZnCl₂, 50% (v/v) glycerol) and 31 µl sterile water, and placed in liquid nitrogen. The exonuclease III was then inactivated by incubation at 68 °C for 15 min, 1 µl of mung bean nuclease (Promega; 3 units µl⁻¹) was added and the mixture was incubated at 30 °C for 30 min. After the incubation, 1.5 µl 10% (w/v) SDS, 2 µl 1 M-Tris/HCl pH 8.6, 4 µl 8 M-LiCl and 50 µl phenol/chloroform (saturated with 10 mM-Tris/1 mM EDTA, pH 8.0) were added, the mixture vortexed, and then centrifuged for 3 min. The aqueous phase was extracted once with chloroform, and then ethanol-precipitated, washed, dried and resuspended in sterile distilled water. The deleted DNA samples were then ready for analysis on agarose gels, prior to ligation. The extent of deletion was determined by nucleotide sequence analysis.

Nucleotide sequence analysis. Suitable fragments were either subcloned into M13mp18 or M13mp19 (Messing, 1983) or sequenced

directly from recombinant plasmids using supercoiled plasmid templates. Supercoiled template preparation was essentially as described by Kraft *et al.* (1988), and templates were denatured as described by Lim & Pen (1988). Nucleotide sequence analysis was performed by the dideoxy-chain termination method using a modified T7 DNA polymerase (Tarbor & Richardson, 1987). Sequencing reactions were electrophoresed on 6% (w/v) denaturing polyacrylamide gels, which were cast as wedge gels with a thickness gradient of 0.2 mm (top) to 0.6 mm (bottom) (Olsson *et al.*, 1984).

Detection of plasmid encoded protein products by maxicell analysis. Proteins encoded by cloned DNA fragments were specifically labelled with [³⁵S]methionine using 'maxicells', and radiolabelled proteins were analysed by denaturing PAGE (Calhoun & Grey, 1981).

Preparation of cell-free extracts and enzyme assay. Cells containing the appropriate plasmid were grown to stationary phase in LB medium (typically 500 ml) containing ampicillin. Following growth, cells were harvested by centrifuging at 6000 g for 10 min and resuspending the pellet in 200 ml 50 mM-Tris/HCl buffer, pH 8.0. The cells were again collected by centrifugation at 6000 g for 10 min and the pellet was resuspended in a small volume (5–7 ml) of ice-cold 50 mM-Tris/HCl buffer pH 8.0. Each sample of washed cells was then disrupted by ultrasonication on ice, and the sonicates were centrifuged at 4 °C for 20 min at 10000 g. The supernatants were assayed for enzyme activity using a method based on that of Winkler & Stuckmann (1979). Assays were carried out by adding 100 µl cell-free extract, 25 µl substrate solution (9 mM in isopropanol) and 975 µl assay buffer (50 mM-Na₂HPO₄, 5.75 mM-sodium deoxycholate and 0.11% gum arabic, pH 8.0) prewarmed to 37 °C. Rates of hydrolysis of substrates by cell-free extracts were measured on an Hitachi recording spectrophotometer at 37 °C. In all cases, rates of hydrolysis of substrates by the control extracts were subtracted from sample rates; *p*-nitrophenylbutyrate and *p*-nitrophenylacetate were found to spontaneously hydrolyse at a significant rate in aqueous solution (0.005 nmol min⁻¹). Specific activity was expressed as nmol substrate hydrolysed per minute per mg of protein present in the cell-free extract. Protein was estimated using a bicinchoninic assay reagent kit (Pierce).

Results and Discussion

Isolation of the *estA* gene

DNA from LS107d2 was used to construct a cosmid library, from which 2000 colonies were screened on BFA and TBA plates. After 10 d incubation, nine positive colonies were detected on TBA and none on BFA. Cosmid DNA was isolated from the most active TBA clone, pLGB1, for further analysis. The cosmid was subcloned by partial digestion with *Sau3A* followed by ligation with *Bam*HI-digested pHP34. Recombinants were then patched on media containing TBA to detect active subclones. Two positive subclones, pLGB11 and pLGB12 (see Fig. 1a), were selected for further

Fig. 1. (a) Summary of cosmid bank construction and initial subcloning of the *estA* coding region. Vector sequence, pHP34 (Prentki & Krisch, 1982), in the plasmid maps is represented by a thin black line. The cross-hatched line represents insert DNA. The *bla* gene and origin of replication are represented by arrows. (b) Summary of subcloning to define the *estA* coding region. Vector sequences, pHP34 and pSK (M13+), in the plasmid maps are represented by thin and thick black lines, respectively. The cross hatched line represents insert DNA. The extent and orientation of the *estA* coding sequence is represented by an arrow.

1 GTAAAAGCAACCTGGCGGCGGTGGACCAACTGGGCAAGGAGCGGAAAAGGCCAAGGCGGCAAGCGCTGTC
 72 CGTGAAGGTGAAGGCGGTAAGTCTGGGCATGACGGCAAAGGCGCAGGGCAGGGCGCCGGGCATGCCGGTGG
 SD
 143 GCACTCCGCCAACGGTCATTGATTGATCGCTGACGGGTTACAAAACTACAAGGGCTGGAACAATGTCCG
 214 CCACTTTTCA ATG GTG TTT TTC GAG GCG GCT TAC GTG CAG ATT CAG GGT CAT TAC
 1 Met Val Phe Phe Glu Ala Ala Tyr Val Gln Ile Gln Gly His Tyr
 AsuII
 268 GAA CTC CAG TTC GAA GCG ATA CGT GAA GCG TTT GCC GCG CTG TTC GAT GAC CCA
 16 Glu Leu Gln Phe Glu Ala Ile Arg Glu Ala Phe Ala Ala Leu Phe Asp Asp Pro
 322 CAG GAG CGT GGC GCC GGG CTT TGC ATT CAG ATC GGC GGC GAA ACC GTG GTC GAT
 34 Gln Glu Arg Gly Ala Gly Leu Cys Ile Gln Ile Gly Gly Glu Thr Val Val Asp
 376 CTG TGG GCC GGC ACC GCT GAC AAG GAC GGT GCC GAG GCC TGG CAC AGC GAC ACC
 52 Leu Trp Ala Gly Thr Ala Asp Lys Asp Gly Ala Glu Ala Trp His Ser Asp Thr
 430 ATC GTC AAT CTT TTC TCC TGC ACC AAG ACA TTT ACT GCC GTC ACG GCC CTG CAA
 70 Ile Val Asn Leu Phe Ser Cys Thr Lys Thr Phe Thr Ala Val Thr Ala Leu Gln
 Scal
 484 TTG GTG GCT GAG GGT AAG TTG CAG TTG GAC GCT CCA GTT GCC AAG TAC TGG CCG
 88 Leu Val Ala Glu Gly Lys Leu Gln Leu Asp Ala Pro Val Ala Lys Tyr Trp Pro
 538 GAA TTT GCC GCT GCC GGT AAG GAA AGC ATC ACG CTG CGC CAG TTG CTC TGC CAT
 106 Glu Phe Ala Ala Ala Gly Lys Glu Ser Ile Thr Leu Arg Gln Leu Leu Cys His
 592 CAG GCG GGT TTG CCG GCG ATC CGC GAG ATG CTG CCC ACC GAG GCC CTG TAT GAC
 124 Gln Ala Gly Leu Pro Ala Ile Arg Glu Met Leu Pro Thr Glu Ala Leu Tyr Asp
 Sall
 646 TGG CAA CTG ATG GTC GAC ACC CTC GCG GCC GAA GCA CCC TGG TGG ACG CCG GGC
 142 Trp Gln Leu Met Val Asp Thr Leu Ala Ala Glu Ala Pro Trp Trp Thr Pro Gly
 700 CAA GGC CAT GGT TAC GAG GCG ATC ACC TAC GGC TGG CTG GTC GGC GAA CTG CTG
 160 Gln Gly His Gly Tyr Glu Ala Ile Thr Tyr Gly Trp Leu Val Gly Glu Leu Leu
 754 CGC CGC GCC GAT GGG CGT GGG CCT GGT GAA TCC ATT GTG GCG CGC GTT GCA CGG
 178 Arg Arg Ala Asp Gly Arg Gly Pro Gly Glu Ser Ile Val Ala Arg Val Ala Arg
 808 CCG TTG GGC CTG GAC TTT CAT GTG GGC CTG GCG GAT GAA GAG TTT TAT CGT GTT
 196 Pro Leu Gly Leu Asp Phe His Val Gly Leu Ala Asp Glu Glu Phe Tyr Arg Val
 862 GCC CAT ATA GCG CGC AGC AAA GGC AAT ATG GGC GAT GAA GCT GCG CAA CGT TTA
 214 Ala His Ile Ala Arg Ser Lys Gly Asn Met Gly Asp Glu Ala Ala Gln Arg Leu
 916 CTG CAA GTA ATG ATG CGT GAA CCC ACC GCC ATG ACC ACA CGG GCA TTT GCC AAT
 232 Leu Gln Val Met Met Arg Glu Pro Thr Ala Met Thr Thr Arg Ala Phe Ala Asn
 Scal
 970 CCA CCG TCT ATT TTG ACC AGT ACT AAT AAA CCT GAA TGG CGA CGC ATG CAG CAG
 250 Pro Pro Ser Ile Leu Thr Ser Thr Asn Lys Pro Glu Trp Arg Arg Met Gln Gln
 SacII
 1024 CCC GCG GCT AAT GGT CAC GGT AAT GCG CGT AGC CTG GCG GGT TTT TAT AGT GGT
 268 Pro Ala Ala Asn Gly His Gly Asn Ala Arg Ser Leu Ala Gly Phe Tyr Ser Gly
 1078 TTG TTG GAC GGT AGT TTG TTG GAA GCC GAC ATG CTC GAA GAG TTG ACC CGT GAA
 286 Leu Leu Asp Gly Ser Leu Leu Glu Ala Asp Met Leu Glu Glu Leu Thr Arg Glu
 1132 CAC AGT ATC GGG CCG GAT AAA ACA TTA TTG ACA CAA ACT CGC TTT GGC TTG GGC
 304 His Ser Ile Gly Pro Asp Lys Thr Leu Leu Thr Gln Thr Arg Phe Gly Leu Gly
 1186 TGC ATG TTG GAT CAA CCG CAG ATG CCT AAT GCC ACA TTC GGC CTT GGC CCG CGT
 322 Cys Met Leu Asp Gln Pro Gln Met Pro Asn Ala Thr Phe Gly Leu Gly Pro Arg
 1240 GCT TTT GGG CAT CCT GGT GCA GGT GGA TCG GTA GGT TTT GCC GAC CCC GAA CAC
 340 Ala Phe Gly His Pro Gly Ala Gly Gly Ser Val Gly Phe Ala Asp Pro Glu His

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1294  GAT GTA GCG TTT GGT TTC GTG ACT AAT ACA TTG GGG CCT TAT GTA CTT ATG GAC
358 ▶ Asp Val Ala Phe Gly Phe Val Thr Asn Thr Leu Gly Pro Tyr Val Leu Met Asp
                                pLGBe320                                pLGBe271
1348  CCT CGT GCA CAG AAG TTG GTC GGA ATA TTG GCC GGT TGT CTG TAA ACCCCTTGCTG
376 ▶ Pro Arg Ala Gln Lys Leu Val Gly Ile Leu Ala Gly Cys Leu ...
                                pLGBe274
1404  TTTCACATTTTTTTTGTACAAAGGCAACTATAAGAAGACGCTGAACGAAATGATGTAACCTTTAATGTTCTG
                                EcoRV
1475  TAAGCGTCTGTTTAAACGGGTCACTCCAGACCCCGTTTCTTTTCTCATTTTGTGGATATCTCATGTTATCGA
1546  ACAAGTCCTTGGCACTGGCGCTTTGCCTCACCATTACCGGCTGCGCACAAACTCCACAGAATGATGCCGAG
1617  GGTGGGCATTGGTGGTCATTTGGATCTGATAAGGCCGCTACCAAGGACGTCGTGACCCAGACCGAGGCCAA
1688  GCCGGATGCCAAACCCGAGTTGGCGCCAAGCCTGCCGCACCTGTAGCCGCTGCGCCTGCTCCGGCAGCCAA
1759  A

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Fig. 2. Nucleotide sequence of the *estA* coding region. Nucleotide and derived amino acid sequences are numbered on the left. The proposed Shine–Dalgarno sequence (SD) is underlined. Relevant restriction sites are marked above the sequence. Also noted above the sequence are the plasmids containing deletions created by exonuclease III (see also Fig. 1*b*) from the *EcoRV* site into the *estA* coding region. The deletions extend to, but do not include, the nucleotide residues shown in bold in the nucleotide sequence.

subcloning to define the coding region. Both pLGB11 and pLGB12 were digested with *PvuII* followed by self ligation. The resulting plasmids, pLGB121 and pLGB111 (see Fig. 1*a*), both retained activity on TBA. To facilitate sequence determination, an *EcoRV* fragment from pLGB121 was cloned in both orientations into the *EcoRV* site of pSK to give pLGBe and pLGBd (see Fig. 1*b*). Both orientations gave detectable activity on TBA; however, pLGBd was considerably more active than pLGBe, indicating that a vector promoter may be involved in expression. Since later studies (see below) indicate that the gene product is an esterase, the gene has been designated *estA*.

Nucleotide sequence of the *estA* region

The nucleotide sequence of the *estA* gene and its flanking regions was determined entirely on both strands using templates generated by one of three methods: (1) When sequencing regions were not contained within pLGBe or pLGBd, fragments from pLGB111 were cloned into M13 vectors; (2) pLGBd and pLGBe were digested with restriction enzymes and self-ligated; or (3) overlapping unidirectional deletions of pLGBe were created using exonuclease III and mungbean nuclease. The nucleotide sequence of the *estA* coding region is shown in Fig. 2. An ORF encoding a polypeptide of 389 amino acid residues with a calculated molecular mass of 42276 Da initiates with a methionine codon at nucleotide position 224, and terminates with a TAA codon at nucleotide position 1393. A notably weak Shine–Dalgarno (SD) sequence, comprising only two bases, is present, appropriately spaced, 9 bp upstream from the initiating methionine

residue (see Gold *et al.*, 1981). Codon usage analysis of *estA* (data not shown) reveals a preference for synonymous codons with C or G residues in the third position, which is particularly evident in the case of codons for ala, leu and cys. This is consistent with the high genomic G+C content in pseudomonads (West & Iglewski, 1988). Comparison of *estA* with sequences in GenBank did not reveal any significant similarities at the nucleotide or amino acid levels. Also, no significant similarity is evident between the deduced amino acid sequence of the *estA* gene product and that of an esterase from an isolate of *Pseudomonas fluorescens* (Choi *et al.*, 1990).

Definition of the coding region of the *estA* gene

Because of the convenient orientation of the polylinker in pLGBe, deletions of this plasmid (initially constructed for sequencing) were used to define the coding region of the *estA* gene. TG1 was transformed with either pLGBe or deletions of pLGBe, plated onto TBA and incubated at 30 °C for 4–8 d. Activity was scored as a clear halo around individual colonies. The results of these deletions on activity are presented in Fig. 1*b*. Deletions outside the coding region near the 3' end of the ORF (pLGBe274 and pLGBe271) do not result in any loss of activity compared to the undeleted plasmid (pLGBe). Cells containing pLGBe320 are EstA⁺, as are cells containing pLGBeSS, formed by deleting from the *SacII* site in the ORF to the *SacII* site in the polylinker. Similarly, a deletion in the 5' region of the ORF, from *AsuII* to the *Clal* site in the polylinker (to produce pLGBeCA), also results in a total loss of activity. These deletions are

Fig. 3. SDS-PAGE analysis of plasmid-encoded polypeptides labelled by the incorporation of [35 S]methionine using maxicells. Samples were electrophoresed through a 12% (w/v) polyacrylamide gel which was then dried and fluorographed. The exposure time was 20 h. Bars on the left side of the figure represent the position of 125 I-labelled molecular mass standards. Top arrow indicates the position of the *estA* gene product, molecular weight 42 kDa (calculated from mobilities of labelled standards). Bottom arrow indicates the position of β -lactamase, molecular weight 30 kDa. Lanes: 1, 125 I-labelled standards; 2, extract of maxicells containing pLGBd, containing *estA* (see Fig. 1b); 3, extract of maxicells containing a control plasmid (pSK containing an insert of non-bacterial origin).

therefore consistent with the ORF derived from the nucleotide sequencing data.

Further evidence on the identification of the ORF was obtained by selectively labelling plasmid-encoded proteins with [35 S]methionine using the maxicell procedure (Fig. 3). A single polypeptide was observed with a molecular mass of 42 kDa, consistent with the molecular mass of 42276 Da calculated from the ORF.

(v/v) glycerol/phosphate buffer, pH 7.0, and stored at -20°C ; cell-free extracts (prepared as described in Methods) were derived from cells grown at 25°C . (a) Lipase from *Pseudomonas* sp. (Boehringer), (b) esterase from rabbit liver (Sigma), (c) cell-free extract from *E. coli* (*lipA*), (d) cell-free extract from *E. coli* (*estA*). Cell-free extracts from *E. coli* containing a control recombinant plasmid gave a small level of activity in the nitrophenol substrates used (corresponding to between 0.003 and 0.08 nmol min^{-1} (mg protein^{-1})) and were subtracted from sample rates. Data are representative of at least three independent experiments, activities are the means of triplicate determinations. Abbreviations: P-ac, *p*-nitrophenylacetate; P-but, *p*-nitrophenylbutyrate; O-but, *o*-nitrophenylbutyrate; P-my, *p*-nitrophenylmyristate; P-pal, *p*-nitrophenylpalmitate; P-ste, *p*-nitrophenylstearate.

Fig. 4. Effect of solubility on nitrophenol ester hydrolysis by either previously characterized enzyme preparations, or *E. coli* extracts containing *estA* or *lipA*. Hydrolysis was measured by nitrophenol release and specific activities were calculated as described in the text. Previously characterized enzyme preparations were suspended in 50%

Characterization of the *estA* gene product

In this investigation, 1% (v/v) tributyrin plates were used to screen for enzymes that may be responsible for the production of short-chain fatty acids which cause rancidity in milk. Since tributyrin is partially soluble (0.01%; Brockerhoff, 1969) in water, the enzyme responsible for clearing tributyrin in solid media (see Methods) could be either an esterase (acting on the soluble component of the substrate) or a lipase (acting on the insoluble portion). To further characterise the *estA* gene product, cells containing pLGBd were grown on media containing 0.1% triolein (a completely insoluble compound) together with cells containing a plasmid which contains a gene (*lipA*) which encodes a lipase (unpublished results) derived from the same cosmid library as pLGBd. Only those cells containing *lipA* gave noticeable clearance of triolein after 3–4 d at 30 °C (data not presented). Since the *lipA* gene product was able to act on an insoluble substrate, the enzyme responsible can therefore be identified as a lipase. However, the inability of the *estA* gene product to hydrolyse triolein may be due to reasons other than the solubility of the substrate. We have therefore compared the activity in cell-free extracts using soluble and insoluble substrates. For comparison, we have also examined a previously characterized esterase and lipase, obtained from commercial sources. Fig. 4(a) shows that water-insoluble compounds (*p*-nitrophenylmyristate, *p*-nitrophenylpalmitate and *p*-nitrophenylstearate) are all substrates for a lipase derived from a species of *Pseudomonas*, whereas only one water-soluble compound (*p*-nitrophenylbutyrate) is a substrate for this enzyme; this result is consistent with the enzyme being a lipase, since other lipases have been shown to hydrolyse a few water-miscible esters (Brockerhoff, 1969). In contrast, esterase from rabbit liver only gave significant rates of hydrolysis with the water-soluble substrates *p*-nitrophenylbutyrate and *o*-nitrophenylbutyrate (Fig. 4b). Consistent with observations that cells containing *lipA* can hydrolyse triolein (a water-insoluble substrate), Fig. 4(c) shows that extracts of cells containing *lipA* hydrolyse the water-insoluble esters of nitrophenol in preference to the water-soluble substrates. In marked contrast, extracts of cells containing *estA* only gave significant rates of hydrolysis with the water-soluble substrates (*p*-nitrophenylbutyrate, *p*-nitrophenylacetate and *o*-nitrophenylbutyrate), and not with the insoluble substrates; this result suggests that the *estA* gene codes for an esterase (Fig. 4c).

The characterization of *estA* will allow a determination of its possible role in the generation of fatty acids from milk triglycerides. For example, *estA* mutants of *Pseudomonas* LS107d2 may be constructed by site-

directed mutagenesis using the cloned gene. Also, studies on the specificity of the *estA* gene-product will be aided by expression in *E. coli*.

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