Molecular analysis and expression of the extracellular lipase of *Aeromonas hydrophila* MCC-2

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The structural gene encoding the extracellular lipase of *Aeromonas hydrophila* MCC-2 was cloned and found to be expressed in *Escherichia coli* using its own promoter. When the cloned gene (*lip*) was expressed in *E. coli* minicells, an 80 kDa protein was identified. Subcellular fractionation of *E. coli* carrying the *lip* gene indicated that the Lip protein was mainly associated with the membrane fraction. Nucleotide sequence analysis revealed that the gene is 2253 bp long, coding for a 79.9 kDa protein with an estimated pI of 10.36. The deduced protein contains two putative signal peptide cleavage sites; one is a typical signal peptidase cleavage site and the other bears a strong resemblance to known lipoprotein leader sequences. Radioactivity from [³H]palmitate was incorporated into the Lip protein when expressed in *E. coli*. The deduced protein contains a sequence of VHFLGHSLGA which is very well conserved among lipases. It shows 67% and 65% overall identity to the amino acid sequences of lipase from *A. hydrophila* strains H3 and JMP636, respectively, but shows little homology to those of other lipases. The Lip protein was purified to homogeneity from both *A. hydrophila* and recombinant *E. coli*. In hydrolysis of *p*-nitrophenyl esters and triacylglycerols, using purified enzyme, the optimum chain lengths for the acyl moiety on the substrate were C₁₀ to C₁₂ for ester hydrolysis and C₈ to C₁₀ for triacylglycerol hydrolysis.

Keywords: lipase, *Aeromonas hydrophila*, lipoprotein

INTRODUCTION

The Gram-negative bacteria belonging to the genus *Aeromonas* are ubiquitous, waterborne micro-organisms that have been recognized with increasing frequency as human and fish pathogens. Several reports have implicated *Aeromonas* species, particularly *A. hydrophila*, as a cause of intestinal and extraintestinal infections in humans (Agger *et al.*, 1985; Figura *et al.*,

1986; Janda & Duffey, 1988; Altwegg & Geiss, 1989; Mikhail *et al.*, 1990; Namdari & Bottone, 1990; Ko & Chuang, 1995). The exact mechanism for the pathogenesis of *A. hydrophila* is not presently understood. Several potential virulence factors such as enterotoxins (Burke *et al.*, 1981; Asao *et al.*, 1984; Rose *et al.*, 1989), haemolysins (Johnson & Lior, 1981; Kuijper *et al.*, 1989), cytotoxic proteins (Lawson *et al.*, 1985), a surface protein (Dooley *et al.*, 1988) and flexible pili (Ho *et al.*, 1990) related to the pathogenicity of *A. hydrophila* infections have been described. However, *A. hydrophila* is also known to secrete a number of degradative enzymes, such as proteases (Leung & Stevenson, 1988), DNase (Chang *et al.*, 1992b), RNase (Favre *et al.*, 1993) and lipases (Anguita *et al.*, 1993). It has been suggested that lipases may provide bacterial nutrients (Hedstrom & Nissson, 1975) and constitute virulence factors by interacting with human leukocytes (Rollof *et al.*, 1988) or by affecting several immune system functions through

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The GenBank accession number for nucleotide sequence data reported in this paper is U63543.

free fatty acids generated by lipolytic activity (Buttke & Cuchens, 1984; Eftimiadi *et al.*, 1987). In addition, a number of potential industrial applications for microbial lipases have been developed (Harwood, 1989). Consequently, the use of these enzymes in industry is expected to increase.

In the present work, we have undertaken the biochemical characterization and the genetic study of an extracellular lipase from *A. hydrophila*. The properties of this lipase are compared with those of the other lipases previously characterized from different strains of *A. hydrophila*.

METHODS

Bacterial strains, plasmids and cultivation condition. *A. hydrophila* MCC-2 was used in this study. It was initially isolated from a blood culture of a hospitalized patient and identified by the Culture Collection and Research Center, Food Industry Research and Development Institute, Hsinchu, Taiwan, Republic of China. *Escherichia coli* JA221 (Beggs, 1978) and *E. coli* JM109 (Yanisch-Perron *et al.*, 1985) were used as hosts for recombinant plasmids. *E. coli* P678-54 (F⁻ *thr leu thi supE lacY fhuA gal mal xyl mtl*) was used as minicell-producing strain. For labelling of proteins with [³H]palmitate, *E. coli* M15 (F⁻ Nal^s Str^s Rif^s *lac ara gal mtl*) (QIAGEN) was employed. Plasmid pBR322 (Bolivar *et al.*, 1977) was used in cloning experiments; plasmids pUC18 and pUC19 (Vieira & Messing, 1982) were used in subcloning experiments.

A. hydrophila and *E. coli* were usually grown in LB medium (Sambrook *et al.*, 1989) at 30 °C and 37 °C, respectively. For screening lipolytic clones, tributyrin plates containing 0.4% caso agar (Merck), 0.1% gum arabic, 1.0% (v/v) tributyrin, 50 µg ampicillin ml⁻¹ and 1.5% agar were used. When necessary, the medium was supplemented with IPTG to a final concentration of 1 mM.

Construction and screening of the DNA library. All DNA manipulations were carried out in accordance with the manufacturers' instructions and as described by Sambrook *et al.* (1989). The preparation of the DNA library containing *A. hydrophila* genomic DNA in the vector pBR322 has been described previously (Chang *et al.*, 1993). The *A. hydrophila* MCC-2 genomic DNA library was constructed in a similar manner. The gene bank was used to transform *E. coli* JA221 and transformants were selected on tributyrin plates. After 48 h incubation at 37 °C, the colonies that had clear zones around them were isolated.

Enzyme activity measurements. Esterase activity was assayed spectrophotometrically using *p*-nitrophenyl butyrate as substrate. The reaction was carried out at 37 °C in 20 mM phosphate buffer (pH 7.4) containing 0.2% Triton X-100, 5 mM *p*-nitrophenyl butyrate, and enzyme. The reaction was stopped with methanol and the absorbance at 420 nm recorded. One unit of esterase activity is defined as the activity which liberates 1 µmol *p*-nitrophenol min⁻¹ under the assay conditions used. Substrate specificity was measured using *p*-nitrophenyl esters of different chain lengths (purchased from Sigma), which were emulsified completely by sonication in the presence of 0.5% Triton X-100.

Lipase activity was measured by the titrimetric method as described by Anguita *et al.* (1993), with tributyrin emulsions used as substrate. One unit of lipase activity is defined as the activity which liberates 1 µmol free fatty acids min⁻¹ under the

assay conditions used. Phospholipase activity was tested by the method described by Ingham & Pemberton (1995). Substrate specificity was measured using various triacylglycerol emulsions (purchased from Sigma). During purification, the spectrophotometric method was used for tracing of lipase activity, with *p*-nitrophenyl butyrate as substrate. β -Galactosidase and β -lactamase activities were assayed as described previously (Chang *et al.*, 1992b). Protein concentrations were determined using a protein assay kit (Bio-Rad) with bovine serum albumin as standard.

Minicell isolation and labelling of proteins. Minicells were isolated from *E. coli* P678-54 containing the appropriate plasmid, and proteins were labelled with [³⁵S]methionine by methods described previously (Doagan & Kehoe, 1984; Chang *et al.*, 1992a). The labelled plasmid-encoded proteins were separated by SDS-PAGE (Laemmli, 1970) and identified by direct autoradiography of the dried gel, using X-ray film.

Minicell fractionation and cellular fractionation. Labelled minicells were collected and converted to spheroplasts by the method of van Doorn *et al.* (1982). The periplasmic proteins will remain in the supernatant when the spheroplasts are pelleted. The spheroplasts were lysed by five cycles of freezing-thawing. After removal of unlysed spheroplasts and minicells, the total membrane (pellet) and cytoplasmic fractions (supernatant) were collected by centrifugation (15 000 g, 30 min). The proteins present in the various fractions of minicells were separated by SDS-PAGE (Laemmli, 1970). Extracellular, periplasmic and cytoplasmic cell fractions of *E. coli* JM109 were prepared as described previously (Chang *et al.*, 1993). The total membrane of *E. coli* was further separated into cytoplasmic and outer membrane fractions with a discontinuous sucrose gradient (Gennity *et al.*, 1992) for unlabelled cells or with sodium lauryl sarcosinate (Filip *et al.*, 1973) for [³⁵S]methionine-labelled cultures.

DNA sequencing and sequence analysis. All sequences were determined on denatured dsDNA templates by the dideoxy chain-termination method (Sanger *et al.*, 1977), using a Sequenase 2.0 DNA sequencing kit (USB) and [³⁵S]dATPαS sequencing grade (> 12.5 mCi ml⁻¹, > 463 MBq ml⁻¹; sp. act. 1000–1500 Ci mmol⁻¹, 37–55.5 TBq mmol⁻¹) (New England Nuclear). Universal and reverse primers were used to obtain the initial sequences within the inserts, and then specific primers for the sequences within the inserts were generated. Conditions for DNA sequencing were as described in the Sequenase brochure (USB). Denaturing 6% polyacrylamide PAGE at 60 W was used to separate the reaction products. The gels were exposed to X-ray film (Kodak Omat-AR) overnight. DNA was sequenced in both directions. In some cases, dITP was used in place of dGTP for a more reliable reading through regions of high G + C content. Sequence analysis was carried out with a PC/GENE software package (Intelligenetics).

Construction of lip expression plasmid pQEL1. Two DNA oligonucleotide primers were designed to allow the PCR amplification of the entire *lip* gene from plasmid pLP12. The forward primer (5'-AGCATGCGAAGAAAAAGCTAATTT-ACGC-3') and the reverse primer (5'-TGCTTAGCAGGTAC-CCGTCGGTCA-3') were synthesized (see Fig. 3). The forward primer contained a *Sph*I restriction site and showed sequence identity with nucleotides 547 to 574 of the *lip* gene, and the reverse primer contained a *Kpn*I site and contained a sequence complementary to nucleotides 2805 to 2828 of the *lip* gene. PCR amplification was performed with 50 pmol of each primer, 200 µM of each deoxynucleoside triphosphate and 2 ng of plasmid pLP12 DNA as template in a total volume of 50 µl. *Taq* polymerase was obtained from Amersham and was used under buffer conditions recommended by the manu-

facturer. PCR was carried out for 30 cycles as described by Innis & Gelfand (1990). The PCR product was purified by phenol/chloroform extraction followed by ethanol precipitation and a wash with 70% ethanol; it was then dried and resuspended in 30 μ l distilled water. The DNA was then cleaved with *Sph*I and *Kpn*I and run on a 0.7% agarose gel in TBE buffer (Sambrook *et al.*, 1989). DNA bands stained with ethidium bromide were visualized under long-wave length UV light and cut from the gel. The vectors, pQE-50, -51, -52 (QIAexpress kits purchased from QIAGEN), were prepared by cleaving 2 μ g vector DNAs with *Sph*I/*Kpn*I and cutting the linearized DNAs band from the gel. About 200 ng insert DNA was ligated to 100 ng vector DNAs and the ligated plasmids were used to transform competent cells of *E. coli* M15(pREP4) (supplied in the QIAexpress kits), which were then plated on LB plates containing 100 μ g ampicillin ml^{-1} and 25 μ g kanamycin ml^{-1} . After overnight incubation at 37 °C, colonies were selected on tributyrin plates to screen for the correct insertion of the coding fragment. The resulting plasmid, pQEL1, was rescued as described by Sambrook *et al.* (1989).

Labelling of proteins with [^3H]palmitate. *E. coli* M15 containing pQEL1 and pREP4 was grown in LB broth supplemented with 100 μ g ampicillin ml^{-1} and 25 μ g kanamycin ml^{-1} . The cells were grown to exponential phase and [^3H]palmitate (5 mCi ml^{-1} ; 185 MBq ml^{-1}) (DuPont-NEN) and IPTG were added to a final concentration of 25 $\mu\text{Ci ml}^{-1}$ and 5 mM, respectively, and incubation was continued for 5 h. The cells were collected by centrifugation and washed three times with methanol to remove noncovalently bound lipids. The dried pellets were resuspended in sample buffer and analysed by SDS-PAGE (Laemmli, 1970). The radio-labelled proteins were detected by autoradiography by using Amplify (Amersham) to enhance the signals and X-ray film.

Purification of lipase and activity-staining. *A. hydrophila* or *E. coli* JM109 harbouring pQEL1 were grown to stationary phase in 2 l LB medium containing 50 μ g ampicillin ml^{-1} , and the extracellular and the membrane fractions were prepared as described above. Lipases from the culture supernatant of *A. hydrophila* and from the *E. coli* cell membrane preparation were purified as described below. All procedures were carried out at 4 °C unless otherwise indicated. The starting material was fractionated with finely powdered ammonium sulfate, and the 25–50% of ammonium-sulfate-precipitated material was resuspended in 50 mM phosphate buffer (pH 7.5) before being applied to a PD-10 column (Pharmacia) to remove salts. The desalting materials were applied to a DEAE-Sephacrose CL-6B column (2.6 $\times$ 15 cm) pre-equilibrated with 50 mM phosphate buffer (pH 7.5). The enzyme was eluted with 500 ml of a linear gradient of 0–1.0 M NaCl in the same buffer. The eluted enzyme fraction was concentrated on a membrane (YM3; Amicon) and then subjected to gel filtration on a Sephadex G-100 column (2.2 $\times$ 100 cm) pre-equilibrated with 50 mM phosphate buffer (pH 7.5). Fractions were eluted at a flow rate of 20 ml h^{-1} in the same buffer, and the highest lipolytic fractions were pooled and applied to a CM-Sephacrose column (1 $\times$ 10 cm) pre-equilibrated with 50 mM phosphate buffer (pH 7.5). The lipase was eluted with 50 ml of a linear gradient of 0–0.5 M NaCl in the same buffer, and the active fractions were collected. Purification was monitored by SDS-PAGE (Laemmli, 1970). Lipase-activity staining was performed as described by Lee *et al.* (1993) with the modification that Tween 80 was used instead of tributyrin as substrate. After electrophoresis, the polyacrylamide gel was laid on 2.0% agarose gel containing 40 mM Tris/HCl (pH 7.8), 50 mM CaCl_2 and 2.0% Tween 80. The attached gel was incubated for 8 h at 37 °C.

RESULTS

Cloning of the *lip* gene of *A. hydrophila*

To clone the gene encoding the extracellular lipase of *A. hydrophila* MCC-2, a genomic library of this organism was constructed in *E. coli* JA221. Transformed *E. coli* cells were screened on tributyrin-containing medium to select tributyrin-degrading clones. Among approximately 5000 transformants, two colonies forming a clear zone were detected. Plasmid DNA was isolated from these two independent clones and subjected to restriction endonuclease mapping. The inserts of the two plasmids (pLP1 and pLP2) were identical, except that the pLP1 insert (7.5 kb) was 2 kb smaller than that of pLP2. To determine the approximate location of the *lip* gene in the cloned DNA fragment, several subclones were generated from pLP1: for each subclone, the ability to grow on tributyrin-containing medium was assayed, and the presence of lipase activity determined. The results of subcloning revealed that a 2.9 kb *Hind*III-*Xho*I fragment was sufficient for the lipase production in *E. coli* JM109 (pLP12) or JM109 (pLP21) (Fig. 1). Since lipase is expressed both from pLP12 and from pLP21, an active promoter on the cloned fragment must transcribe *lip*. In addition, the clear zones formed around colonies containing pLP21 were smaller than those formed around colonies containing pLP12, indicating that the *lacZ* promoter on pLP12 contributes to transcription of *lip*.

Fig. 1. Restriction endonuclease maps of plasmid clones harbouring the lipase gene. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pst*I; S, *Sph*I; X, *Xho*I. Boxes indicate *A. hydrophila* DNA; thin lines indicate DNA from the plasmid vectors pBR322, pUC18 or pUC19; solid arrows indicate the direction of transcription of the tetracycline promoter (pLP1) or the *lacZ* promoter (pLP10, pLP11, pLP12 and pLP21); dashed arrows indicate the direction of the putative promoter of the cloned gene. Varying levels of activity are represented by + and ++.

Fig. 2. ^{35}S -labelled polypeptides from the various fractions of pLP12-containing minicells. Extracts were applied to the gel from whole cells (lane 4), the cytoplasm (lane 3), the periplasmic fluid (lane 2) and total membrane (lane 1). The *lip* gene product is shown by an arrow.

Detection and cellular distribution of lipase in *E. coli* transformants

To identify the product encoded by the cloned *lip* in *E. coli*, pLP12 was transformed into the minicell strain P678-54. Polypeptides encoded by pLP12 and the vector pUC19 were detected by labelling with [^{35}S]methionine. A unique 80 kDa polypeptide was expressed in the strain carrying pLP12 (Fig. 2, lane 4), and the inability to produce the 80 kDa polypeptide was observed in the strain containing pUC19 (data not shown).

Measurement of extracellular lipase activity produced by *A. hydrophila* MCC-2 indicated that activity was very low in exponentially growing cells and the activity increased rapidly in cells of the early stationary phase. Lipase activity was detected neither in the cytoplasm nor in the periplasmic fluids of cells during the exponential growth phase or during the stationary phase (data not shown). To localize the *lip* gene product within *E. coli* P678-54 harbouring pLP12, the labelled minicells were fractionated into periplasmic, cytoplasmic and membrane fractions. SDS-PAGE analysis revealed that the 80 kDa Lip protein was detected exclusively in the membrane fraction of *E. coli* minicells (Fig. 2, lane 1). Enzyme assays revealed that the majority of the lipase activity was also located in the *E. coli* P678-54 membrane fraction (about 70%). When the membrane fraction of *E. coli* P678-54 was further separated into inner and outer membrane fractions utilizing the detergent lauryl sarcosinate, SDS-PAGE analysis showed that the 80 kDa protein was in both fractions (data not shown). To investigate whether this was the case with the cloned Lip in *E. coli* JM109, cells harbouring pLP12 were harvested in early stationary phase and fractionated, and the activity of lipase in these different cell

Table 1. Distribution of lipase and marker enzymes in early stationary phase growth of *E. coli* JM109(pLP12)

Enzyme activities are given as the percentage of the total activity. Experiments were performed in triplicate, and the ranges of values are given.

Fraction	Enzyme activity		
	Lipase	β -Lactamase	β -Galactosidase
Supernatant	5–12	10–15	0–6
Periplasm	5–12	65–80	0–5
Cytoplasm	4–8	4–8	75–85
Membrane	70–85	5–10	10–18

compartments was determined. As controls, β -lactamase (a periplasmic marker enzyme) and β -galactosidase were also assayed for each fraction. The results revealed that β -lactamase was found predominantly in the periplasmic fraction, and β -galactosidase in the cytoplasmic fractions. Most of the lipase activity, however, was detected in the membrane fraction (Table 1).

Nucleotide sequence of *lip* gene

The nucleotide sequence of the 2.9 kb DNA fragment encompassing the *lip* gene was determined on both strands. Computer analysis of the nucleotide sequence identified the ORF for *lip* (Fig. 3), extending from an ATG codon at nucleotide 552 to a TGA stop codon at position 2804, which is sufficient to encode a protein of 751 amino acids corresponding to a molecular mass of 79921 Da. A putative ribosome-binding site (Shine-Dalgarno sequence) was tentatively placed between nucleotide positions 539 and 544 at 7 bp upstream from the ATG. We have not attempted to locate a promoter region upstream of the putative translational start site. A potential transcription termination structure was identified at positions 2845 to 2872. The G + C content of the *A. hydrophila lip* gene (65 mol%) is nearly identical to those for glycerophospholipid-cholesterol acyltransferase (GCAT) (62 mol%; Thornton *et al.*, 1988), α -amylase (63 mol%; Chang *et al.*, 1993), amylase (62 mol%; Gobius & Pemberton, 1988), deoxyribonuclease (DNase) (63 mol%; Chang *et al.*, 1992b), nuclease (64 mol%; Dodd & Pemberton, 1996) and lipase (63 mol%; Anguita *et al.*, 1993), and is a little higher than that for aerolysin (58 mol%; Howard *et al.*, 1987) from this species. The codon usage frequency in the *lip* gene is similar to the pattern for the aforementioned genes of this species (data not shown).

Analysis of the Lip amino acid sequence

Application of a program (von Heijne, 1986) for identification of prokaryotic secretory signal sequences predicted that Lip contains a signal peptidase cleavage site between residues 48 and 49 (Ala-Ala). However, we also found a second possible cleavage site between

[illegible]

Fig. 3. Nucleotide and deduced amino acid sequences of the *lip* gene of *A. hydrophila*. Numbers refer to the nucleotide or amino acid located at the end of each line. The putative Shine-Dalgarno sequence is underlined. The lipase consensus sequence at positions 518 to 527 is shown. The putative palindromic transcription termination structure (positions 2845 to 2872) is also underlined. The asterisks after the 17th and 48th amino acids indicate the two potential leader peptide cleavage sites.

residues 17 and 18 (Gly-Cys). This second site has a strong resemblance to known lipoprotein signal peptide sequences when aligned with the cysteine residue as +1 (Wu & Tokunaga, 1986). The unprocessed protein appears to be highly basic, with an estimated pI of 10.36, and contains nine Cys residues, some of which may form disulfide bonds important in the secondary structure of the protein. Additionally, the enzyme contains the sequence Val-His-Phe-Leu-Gly-His-Ser-Leu-Gly-Ala (positions 518 to 527), which matches almost perfectly the consensus sequence of lipases of different origins and with different biochemical properties. It is generally supposed that this consensus sequence represents the substrate-binding domain of lipase enzymes (Antonin, 1988) and that the central serine of the conserved

Lip	MKKLLIYAAYVAVSALLSGCGGSDDNKGDTSYSLDYLLSGSNVAVRPSAALAAR	50
H3	MKKLLIYAAYVAVSALLSGCGGSDDNKGDTSYSLDYLLSGSNVAVRPSAALAAR	50
Ap11	MKKNIYAAYVAVSALLSGCGGSDDNKGDTSYSLDYLLSGSNVAVRPSAALAAR	50

Lip	ATDGTLLKFSTETADLSNPVVSALSTLQGWSTTQAIQIVPVTS-GIQVKAPA	99
H3	ASDGTLLKFSTETADLSNPVVSAMSTLQGWSTTQAIQIVPVTSSTGIQIVQAT	100
Ap11	PSDGTLLKFSTETADLSNPVVSALSTLQGWSTTQAIQIVPVTSASGITVQAT	100

Lip	AAEFTASVAPLYLMELEFDSAAALRPSGVKKVLYSGVDVFAVEATITROAESG	149
H3	TAEFGASVAPLYLLEVTFDSTALRPSGVKKVLYSGVDVFAVAASWAQAFEG	150
Ap11	TADEFGASVAPLYLLELAFDSTALRPSGVKKVLYSGVDVFAVEASTKAAFG	150

Lip	AADAAPLFL-LLHDSGDRYPQRQHGRPLRPGSDYSNYKTSTSGNSADEOTI	198
H3	SAOAEVPLPLCLANDSGHRTAEROSRRCLKAGSDYGNKYNAGNSADEOTI	200
Ap11	SAOAGQPFLLLLHDSGDRYQARQRRRQPKYKMGSDYSNYKSSPGNSADEOSI	200

Lip	SGLIALQEGELFKAATGITSDHVIFSDNFGTQSGADVLYAVKGAASVLKS	248
H3	NGLIALQEGELFKAATGATGTHVIFSDNFGTQSGADVLYAVKGAASVLKA	250
Ap11	NGLITLQEGELFKAATGITSDHVIFSDNFGTQSGADVLYAVKGAASVLKA	250

Lip	P--TLDAALAKWQDAPGQYOPAHWLYPVGERRPLPDPAAGCRELPQAEQK	296
H3	DPVTLDAALKWQDAWE-HOPARHLYPYGERRPTCLPDPAAGCAVPAAEQK	299
Ap11	RPRTLDAALAEITGCRRA-YOPARYLYPERGYPTPLPEPAGCAVPAASGNK	299

Lip	DAIAAAGFDALHSTIHWVWPVSPASPTSSPPRP-APGTRRRRPSG	345
H3	DAIAATAGFVPLSTRLLRKKRPSIYPVSSCLTSSPHRRPQVAGRPSPSG	349
Ap11	DAIAATAYAPSRPLAEHLRRPRFI-GRSSCLTSSPLRALPIYGTGKPRPSG	349

Lip	TVPSQPLRHRREPE--PGCRSDR--RPGGGG	373
H3	TVPSQPYRHRQCAEGVTSDRRRAGGGGGSGPAGDAPCRSDPPEARAAGRG	399
Ap11	TVPSQPYRHRQCAEGVTSDRRRPGGGRGSGSLAWGTDCRSDSPDRAAGGG	399

Lip	---YGSRDEHRLQPAAGTGRGVRPDAOLRRRTAG--	406
H3	EQADQWDHLRQAAAGRAEHSLOPADADGRGAIADACL--RGCGP	446
Ap11	EQAHRGDPHLGRQAGAGSRAEYRPLQAPAGAGRGAIADAHLCRRRAVC--	447

Lip	EHHRRDDLSARRHLGEGKRLCAGPWN--WCRCQEOERGQGGDRSSLH	453
H3	HHHCCHLSARRLDGRLRRLRPGAGPDNLDLRHAGGEOERGQGGDRSSAA	496
Ap11	-HHRCDHLSAWRHLEGERLCPGAGPD-RCRCSKHHQEGCERGQGGDRSPA	495

Lip	YNA-FALTGSMDSV-TSDNPPYLYNLISLYTVARNLQKSVAIACWACWYR	501
H3	RRAWLRLSGMSDVT-TSDNPPYLYNLISLYTVARNLQKSVAIACWACWYR	545
Ap11	RGAWFRLSGMSDGGDRTTSENPPYLYNLISLYTVARNLQKSVAIACWACWYR	545

Lip	WGWPMPRAIGTAGSLKYVHLGHSLGASRGPTCWWRPNTSLLATRRRRPVQV	551
H3	LAWPTPRAIGTAGSLKYVHLGHSLGASRVPTCCGRQPMWRASGCPYQV	595
Ap11	WGWPMPRAIGTAGSLKYVHLGHSLGASLGPICGRQKPVQWQSPGGCPYQV	595

Lip	RHWRPGHAGRHSAAVAELAAFGPTITQMSVLTAGSAALKAFTAYAPNCKT	601
H3	RYRWPGHAGHSAAAAELADFPTITKGVLTSGSAELKAGTAYAPNCTO	645
Ap11	HHWPGHAGRHSAAAAELTDFGPTIKMVLISGSELPKLTAYAPNCTO	645

Lip	ASTDLLRQRVPAAEPGCGHGHGGHQAELQLRAQSVLSDAPINLGGIR	651
H3	GGAYLRLQRVPAAEPGRGHS-	665
Ap11	RGELLHLQRVPPEPGRYHPA-	665

Lip	GLPAVCHRRNRRLGALSLSORVINSIATAPPQWHRAAVQGGPGPATADRHG	701
H3	---TAATRCRVLQCGPVGAGFG--	684
Ap11	---SAAGFRVMFVPGVRAGFG--	684

Lip	PPTTERRVSWPGVTARCWHRTRTSIRPAPSPRCRPSAASSAAQVPRSR	751
H3	---	684
Ap11	---	684

Fig. 4. Comparison of the deduced amino acid sequences of *A. hydrophila* Lip protein (Lip), H3 lipase (H3) and Apl-1 lipase (Apl1). The amino acid sequence numbers are listed on the right. Asterisks indicate identical residues and dots show conservative substitutions. Gaps introduced for alignment are represented by dashes.

pentapeptide Gly-Xaa-Ser-Xaa-Gly within this sequence is the nucleophilic residue essential for catalysis (Fautinella *et al.*, 1991).

The predicted amino acid sequence of Lip protein was compared with those of other well characterized lipases from *A. hydrophila* strains. Significant divergence was observed between the predicted amino acid sequence of Lip protein and the lipase/acyltransferase, which has been cloned from strain Ah65 (Hilton *et al.*, 1990; data not shown). However, the Lip protein showed strong similarity to the H3 lipase and Apl-1 lipase, which have been cloned from strains H3 and JMP636, respectively (Anguita *et al.*, 1993; Ingham & Pemberton, 1995). Over the entire sequence, the Lip protein was 67 % identical to the H3 lipase and 65 % identical to the Apl-1 lipase. Sequence alignment analysis (Fig. 4) showed that a

Fig. 5. [^3H]Palmitate labelling of lipoproteins from *E. coli* and SDS-PAGE of purified lipase. Lanes 1–3, autoradiograph of SDS-PAGE preparations from cells (carrying pREP4 and pQEL1) labelled with [^3H]palmitate in the presence of IPTG inducer (lane 1), in the absence of IPTG inducer (lane 2) and cells (carrying pREP4) labelled in the presence of IPTG inducer (lane 3). Lane 4, lipase purified from *A. hydrophila* MCC-2 stained with Coomassie blue G250. The arrow indicates the position of Lip protein purified from *E. coli* carrying pQEL1, determined by Coomassie staining of the gel.

major difference between Lip and these two proteins exists, namely that Lip lacks 37 amino acid residues corresponding to Gly-380 to Arg-416 in H3 lipase and Gly-380 to Ser-416 in Apl-1 lipase. In addition, Lip contains an additional 100 amino acids at the C-terminal end which are not present in either H3 lipase or Apl-1 lipase. No other significant similarities were observed with other nucleotide or protein sequences available in the EMBL-GenBank or SWISS-PROT databases.

Post-translational modification of Lip

The presence of a consensus processing site for lipoprotein suggested the possibility that the cloned *lip* gene product was subject to post-translational lipid modifi-

cation when expressed in *E. coli*. To test this possibility, a *lip* expression plasmid, pQEL1, was constructed. *E. coli* M15 harbouring plasmid pREP4 was transformed with pQEL1, and palmitate labelling was carried out (Fig. 5). In addition to labelling of the native *E. coli* lipoproteins, there was substantial labelling of cloned Lip in *E. coli* M15 cells harbouring pQEL1 and pREP4 grown in the presence of [^3H]palmitate and IPTG inducer (Fig. 5, lane 1). No significant labelling of cloned Lip in uninduced *E. coli* M15 harbouring pQEL1 and pREP4 cells, or in *E. coli* harbouring pREP4 cells was seen (lanes 2 and 3).

Purification and characterization of lipase

Cultures of *A. hydrophila* or *E. coli* transformed with pQEL1 were fractionated and the culture supernatants were used as crude preparations for enzyme purification. A typical procedure for purification of the lipase from culture supernatant of *A. hydrophila* MCC-2 is summarized in Table 2. The same protocol was also performed for purification of Lip protein from the membrane fraction of *E. coli* transformed with pQEL1. After examination of both purified lipase preparations by SDS-PAGE, a single band with a molecular mass of 80 kDa was observed (Fig. 5, lane 4), which corresponded to the active lipase bands obtained on a renaturing polyacrylamide gel (Fig. 6). A single active lipase band was present in the crude enzyme preparations from culture supernatant of *A. hydrophila* (Fig. 6, lane 1), indicating that *A. hydrophila* may produce only one extracellular lipase, whose matured form has a molecular mass of 80 kDa.

The optimal activity of the Lip protein was at a pH range of 7.5 to 8.0 when *p*-nitrophenyl butyrate was used as substrate. No activity was detected below pH 5.0 or above pH 9.5. In the optimal pH range, maximal enzyme activity was obtained at 37 °C and the enzyme was stable below 50 °C; after being incubated for 30 min at 55 °C, only 20% of the activity remained. To investigate the substrate specificity of Lip protein, we tested esterase and lipase activities by using *p*-nitrophenyl derivatives

Table 2. Summary of the purification of extracellular lipase from *A. hydrophila* MCC-2.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg ⁻¹)*	Yield	Purification factor
Supernatant	180	2880	1.6	100	1
25–50% ammonium sulfate	47	1645	3.6	57	2.3
DEAE-Sepharose CL-6B	6	81	13.5	28	8.5
Sephadex G-100	0.9	48	53.5	17	33

* One unit is the amount of enzyme which releases 1 μmol *p*-nitrophenol min⁻¹ at 37 °C under the assay conditions used.

Fig. 6. Analysis of lipase activity in crude cell extracts by SDS-PAGE followed by gel renaturation. Lanes: 1, culture supernatants of *A. hydrophila* MCC-2; 2, extracts from *E. coli* (pUC19); 3, extracts from *E. coli*(pLP12). The position of the lipase activity, detected in lanes 1 and 3, is shown by an arrow.

Table 3. Esterase activity of Lip protein against several *p*-nitrophenyl esters and triacylglycerols

Acyl group substrate	Esterase activity of Lip protein against:			
	<i>p</i> -Nitrophenyl derivatives		Triacylglycerols	
	U mg ⁻¹ *	%†	U mg ⁻¹ *	%†
Acetate	0	0	0	0
Butyrate	13.9	12.1	13.5	15.3
Caproate	78.8	68.5	44.1	50.1
Caprylate	86.3	75.0	79.2	90.0
Caprate	115.1	100	88.0	100
Laurate	106.0	92.1	61.6	70.0
Myristate	43.7	38.0	22.0	25.0
Palmitate	0	0	0	0

* Data are means from triplicate determinations.

† Percentage of the maximum activity detected.

and triacylglycerols, respectively. The results (Table 3) show that esterase activity was detected against substrates with chain lengths from C₄ to C₁₄ and the highest esterase activity was observed with the substrate *p*-nitrophenyl caprate (C₁₀). The highest lipase activity was observed with tricaprylin (C₈) and tricaprin (C₁₀) as

substrates. Only 25% of the maximum lipase activity was detected with C₁₄ and no lipolytic activity was observed with C₁₆. To further characterize the Lip protein specificity, we analysed phospholipase activity by using various phospholipids as substrates. No phospholipase activity was detected.

DISCUSSION

The present paper describes the gene encoding the extracellular lipase of *A. hydrophila* MCC-2 and shows that the *lip* gene product is readily expressed in *E. coli* under the control of its own promoter. The Lip protein is encoded by an ORF of 2253 bp. This ORF codes for a protein of 751 amino acids corresponding to a 79.9 kDa polypeptide. The start codon was identified by its location 7 bp downstream from the sequence 5'-AAGAGA-3', which is typical of sequences known to function as ribosome-binding sites in *E. coli* (Shine & Dalgarno, 1974). The translational stop codon TGA, 2253 bp downstream, defines the C-terminal end of the protein and is the preferred stop codon for *A. hydrophila* (Howard *et al.*, 1987; Thornton *et al.*, 1988; Chang *et al.*, 1993; Favre *et al.*, 1993), in contrast to the overall tendency in prokaryotes to be biased towards TAA (Kohli & Grosjean, 1981; Ikemura & Ozeki, 1982).

Although a putative signal sequence was identified, and the predicted cleavage site between Ala and Ala (at positions 48 and 49) is typical of leader sequences of secreted proteins which are cleaved by signal peptidase I (von Heijne, 1986), it was also noted that this cleavage site was preceded by the sequence Leu-Leu-Ser-Gly-Cys, which bears a strong resemblance to the consensus SPase II recognition site of known lipoproteins (Wu & Tokunaga, 1986). Attempts to determine the N-terminal amino acid sequence of the purified recombinant Lip protein were unsuccessful. In contrast, the lipoprotein nature of Lip was seen when *E. coli* M15 cells containing pQEL1 grown in the presence of [³H]palmitate and IPTG inducer were shown to produce ³H-labelled Lip which was not detected in the absence of inducer. Using a bacterial lipoprotein, bacteriocin release protein (BRP), fused to β -lactamase, it was shown that the signal peptide plus the Cys of BRP was sufficient for the lipid modification and processing (Oudega *et al.*, 1993). Similarly, an *in vitro* study using the signal peptide plus four N-terminal amino acids of mature Braun's lipoprotein supported this conclusion (Sankaran & Wu, 1994). Additionally, lipoproteins or lipoprotein intermediates, such as the pullulanase of *Klebsiella pneumoniae* (d'Enfert *et al.*, 1987), pectinesterase of *Pseudomonas solanacearum* (Spok *et al.*, 1991), EGL endoglucanase of *P. solanacearum* (Huang & Schell, 1992) and cel3 endoglucanase of *Fibrobacter succinogenes* (McGavin *et al.*, 1989) contain this consensus cleavage sequence near their N termini and, when produced in *E. coli*, showed evidence of lipid modification and membrane association. Likewise, our results indicated that the Lip protein was primarily membrane-associated and lipid-modified in *E. coli*. Examination of the hydrophathy

profile of this protein and analysis of the amino acid sequence, using the software program PC/GENE, revealed that it is predominantly hydrophilic rather than amphiphilic and contains only one potential transmembrane segment, corresponding to the region of the predicted signal peptide. Therefore, we propose that the lipoprotein consensus sequence in the Lip protein serves as a signal for lipid modification, and the result of the lipid modification is directly responsible for stable association of the protein with the inner face of the outer membrane and the outer face of the inner membrane. This proposal will be tested by site directed mutagenesis of the lipid-modification site.

Since the Lip protein was found to show only limited homology to *A. hydrophila* Ah65 lipase/acyltransferase, which shares several properties with mammalian plasma lecithin-cholesterol acyltransferase (LCAT) (Brumlik & Buckley, 1996), but shows high homology to *A. hydrophila* H3 lipase and JMP636 Apl-1 lipase, it is likely that the Lip protein is more closely related to the latter two lipases. However, there are considerable differences in enzyme properties among the three lipases. Apl-1 lipase possesses phospholipase C activity (Ingham & Pemberton, 1995), while no phospholipase activity was detected in Lip protein. Likewise, molecular sizes, optimum pH, optimum temperature, thermal stability and, especially, substrate specificity of Lip protein and H3 lipase are distinct. The H3 lipase described by Anguita *et al.* (1993) showed high thermostability, and its lipolytic activities were observed with *p*-nitrophenyl derivatives with chain lengths in the range C₄ to C₁₀ as substrates and with triacylglycerols with lengths in the range C₄ to C₈. The Lip protein pI value (10.36) was higher than that of H3 lipase and those of known microbial lipases (Sugiura, 1984; Dartois *et al.*, 1992). In addition, our observation that cloned Lip protein was primarily localized to a membrane-bound site in *E. coli* differs from the findings of Anguita *et al.* (1993). These authors observed that the H3 lipase gene product was present in the periplasmic space in *E. coli*. Based on the amino acid alignment shown, Lip protein contains an additional 100 amino acids at the C-terminal end which are not present in H3 lipase and lacks 37 amino acid residues that correspond to Gly-380 to Arg-416 in H3 lipase. The different enzyme properties described above may result from these major differences of amino acid sequence between the two enzymes. Clearly, further experiments are required to clarify this point.

ACKNOWLEDGEMENTS

This work was supported by research grant NSC84-2331-B006-024 from the National Science Council, Taiwan, Republic of China.

REFERENCES

- Agger, W. A., McCormick, J. D. & Gurwith, M. J. (1985). Clinical and microbiological features of *Aeromonas hydrophila*-associated diarrhea. *J Clin Microbiol* 21, 909–913.
- Altwegg, M. & Geiss, H. K. (1989). *Aeromonas* as a human pathogen. *Crit Rev Microbiol* 16, 253–286.
- Anguita, J., Rodriguez Aparicio, L. B. & Naharro, G. (1993). Purification, gene cloning, amino acid sequence analysis, and expression of an extracellular lipase from an *Aeromonas hydrophila* human isolate. *Appl Environ Microbiol* 59, 2411–2417.
- Antonian, E. (1988). Recent advances in the purification, characterization and structure determination of lipases. *Lipids* 23, 1101–1106.
- Asao, T., Kinoshita, Y., Kozaki, S., Uemura, T. & Sakaguchi, G. (1984). Purification and some properties of *Aeromonas hydrophila* hemolysin. *Infect Immun* 46, 122–127.
- Beggs, J. D. (1978). Transformation of yeast by a replicating hybrid plasmid. *Nature* 275, 104–109.
- Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heyneker, H. L. & Boyer, H. W. (1977). Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* 2, 95–113.
- Brumlik, M. J. & Buckley, J. M. (1996). Identification of the catalytic triad of the lipase/acyltransferase from *Aeromonas hydrophila*. *J Bacteriol* 178, 2060–2064.
- Burke, V., Robinson, J., Berry, R. J. & Gracey, M. (1981). Detection of enterotoxins of *Aeromonas hydrophila* by a suckling-mouse test. *J Med Microbiol* 14, 401–408.
- Buttke, T. M. & Cuchens, M. A. (1984). Inhibition of lymphocyte proliferation by free fatty acids. II. Toxicity of stearic acid towards phytohaemagglutinin-activated T cells. *Immunology* 53, 507–514.
- Chang, M. C., Chang, C. C. & Chang, J. C. (1992a). Cloning of a creatinase gene from *Pseudomonas putida* in *Escherichia coli* by using an indicator plate. *Appl Environ Microbiol* 58, 3437–3440.
- Chang, M. C., Chang, S. Y., Chen, S. L. & Chuang, S. M. (1992b). Cloning and expression in *Escherichia coli* of the gene encoding an extracellular deoxyribonuclease (DNase) from *Aeromonas hydrophila*. *Gene* 122, 175–180.
- Chang, M. C., Chang, J. C. & Chen, J. P. (1993). Cloning and nucleotide sequence of an extracellular α -amylase gene from *Aeromonas hydrophila* MCC-1. *J Gen Microbiol* 139, 3215–3223.
- Dartois, V., Baulard, A., Schanck, K. & Colson, C. (1992). Cloning, nucleotide sequence and expression in *Escherichia coli* of a lipase gene from *Bacillus subtilis* 168. *Biochim Biophys Acta* 1131, 253–260.
- Doagan, G. & Kehoe, M. (1984). The minicell system as a method for studying expression from plasmid DNA. *Methods Microbiol* 17, 233–258.
- Dodd, H. N. & Pemberton, J. M. (1996). Cloning, sequencing, and characterization of the *nucH* gene encoding an extracellular nuclease from *Aeromonas hydrophila* JMP636. *J Bacteriol* 178, 3926–3933.
- Dooley, J. S., McCubbin, W. D., Kay, C. M. & Trust, T. J. (1988). Isolation and biochemical characterization of the S-layer protein from a pathogenic *Aeromonas hydrophila* strain. *J Bacteriol* 170, 2631–2638.
- Eftimiadi, C., Buzzi, E., Tonetti, M., Buffa, P., Buffa, D., Van Steenberghe, M. T., de Graaff, J. & Botta, G. A. (1987). Short-chain fatty acids produced by anaerobic bacteria alter the physiological responses of human neutrophils to chemotactic peptide. *J Infect Dis* 14, 43–53.
- d'Enfert, C., Ryter, A. & Pugsley, A. P. (1987). Cloning and expression in *Escherichia coli* of the *Klebsiella pneumoniae* genes for production, surface localization and secretion of the lipoprotein pullulanase. *EMBO J* 6, 3531–3538.
- Faustinella, F., Smith, L. C., Semenkovich, C. F. & Chan, L. (1991).

- Structural and functional roles of highly conserved serines in human lipoprotein lipase. *J Biol Chem* **266**, 9481–9485.
- Favre, D., Ngai, P. K. & Timmis, K. N. (1993). Relatedness of a periplasmic, broad-specificity RNase from *Aeromonas hydrophila* to RNase I of *Escherichia coli* and to a family of eukaryotic RNases. *J Bacteriol* **175**, 3710–3722.
- Figura, N., Marri, L., Verdiani, S., Ceccherini, C. & Barberi, A. (1986). Prevalence, species differentiation, and toxigenicity of *Aeromonas* strains in cases of childhood gastroenteritis and in controls. *J Clin Microbiol* **23**, 595–599.
- Filip, C., Fletcher, G., Wulff, J. L. & Earhart, C. F. (1973). Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. *J Bacteriol* **115**, 717–722.
- Gennity, J. M., Kim, H. & Inouye, M. (1992). Structural determinants in addition to the amino-terminal sorting sequence influence membrane localization of *Escherichia coli* lipoproteins. *J Bacteriol* **174**, 2095–2101.
- Gobius, K. S. & Pemberton, J. M. (1988). Molecular cloning, characterization, and nucleotide sequence of an extracellular amylase gene from *Aeromonas hydrophila*. *J Bacteriol* **170**, 1325–1332.
- Harwood, J. (1989). The versatility of lipases for industrial uses. *Trends Biochem Sci* **14**, 125–126.
- Hedstrom, S. A. & Nisson, P. (1975). Lipolytic activity of *Staphylococcus aureus* strains from cases of human chronic osteomyelitis and other infections. *Acta Pathol Microbiol Scand Sect B* **83**, 285–292.
- von Heijne, G. (1986). A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res* **14**, 4683–4690.
- Hilton, S., McCubbin, W. D., Kay, C. M. & Buckley, J. M. (1990). Purification and spectral study of a microbial fatty acyltransferase: activation by limited proteolysis. *Biochemistry* **29**, 9072–9078.
- Ho, A. S., Mietzner, T. A., Smith, A. J. & Schoolnik, G. K. (1990). The pili of *Aeromonas hydrophila*: identification of an environmentally regulated 'mini pilin'. *J Exp Med* **172**, 795–806.
- Howard, S. P., Garland, W. J., Green, M. J. & Buckley, J. T. (1987). Nucleotide sequence of the gene for the hole-forming toxin aerolysin of *Aeromonas hydrophila*. *J Bacteriol* **169**, 2869–2871.
- Huang, J. & Schell, M. (1992). Role of the two-component leader sequence and mature amino acid sequence in extracellular export of endoglucanase EGL from *Pseudomonas solanacearum*. *J Bacteriol* **174**, 1314–1323.
- Ikemura, T. & Ozeki, H. (1982). Codon usage and transfer RNA contents: organism-specific codon-choice patterns in reference to the isoacceptor contents. *Cold Spring Harbor Symp Quant Biol* **47**, 1087–1097.
- Ingham, A. B. & Pemberton, J. M. (1995). A lipase of *Aeromonas hydrophila* showing nonhemolytic phospholipase C activity. *Curr Microbiol* **31**, 28–33.
- Innis, M. A. & Gelfand, D. H. (1990). Optimization of PCRs. In *PCR Protocols: a Guide to Methods and Applications*, pp. 3–12. Edited by M. A. Innis, D. H. Gelfand, J. J. Sninsky & T. J. White. San Diego, CA: Academic Press.
- Janda, J. M. & Duffey, P. S. (1988). Mesophilic aeromonads in human disease: current taxonomy, laboratory identification, and infectious disease spectrum. *Rev Infect Dis* **10**, 980–997.
- Johnson, W. M. & Lior, H. (1981). Cytotoxicity and suckling mouse reactivity of *Aeromonas hydrophila* isolated from human sources. *Can J Microbiol* **27**, 1019–1027.
- Ko, W. C. & Chuang, Y. C. (1995). *Aeromonas* bacteremia: review of 59 episodes. *Clin Infect Dis* **20**, 1298–1304.
- Kohli, J. & Grosjean, H. (1981). Usage of the three termination codons: compilation and analysis of the known eukaryotic and prokaryotic translation termination sequences. *Mol Gen Genet* **182**, 430–439.
- Kuijper, E. J., Bol, P., Peeters, M. F., Steigerwalt, A. G., Zanen, H. C. & Brenner, D. J. (1989). Clinical and epidemiologic aspects of members of *Aeromonas* DNA hybridization groups isolated from human feces. *J Clin Microbiol* **27**, 1531–1537.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Lawson, M. A., Burke, V. & Chang, B. J. (1985). Invasion of Hep-2 cells by fecal isolates of *Aeromonas hydrophila*. *Infect Immun* **47**, 680–683.
- Lee, Y. P., Chung, G. H. & Rhee, J. S. (1993). Purification and characterization of *Pseudomonas fluorescens* SIK W1 lipase expressed in *Escherichia coli*. *Biochim Biophys Acta* **1169**, 156–164.
- Leung, K.-Y. & Stevenson, R. M. W. (1988). Characteristics and distribution of extracellular proteases from *Aeromonas hydrophila*. *J Gen Microbiol* **134**, 151–160.
- McGavin, M. J., Forsberg, C. W., Crosby, B., Bell, A. W., Dignard, D. & Thomas, D. Y. (1989). Structure of the *cel-3* gene from *Fibrobacter succinogenes* S85 and characteristics of the encoded gene product, endoglucanase 3. *J Bacteriol* **171**, 5587–5595.
- Mikhail, I. A., Fox, E., Haberberger, R. L., Jr, Ahmed, M. H. & Abbatte, E. A. (1990). Epidemiology of bacterial pathogens associated with infectious diarrhea in Djibouti. *J Clin Microbiol* **28**, 956–961.
- Namdari, H. & Bottone, E. J. (1990). Microbiologic and clinical evidence supporting the role of *Aeromonas caviae* as a pediatric enteric pathogen. *J Clin Microbiol* **28**, 837–840.
- Oudega, B., Clark, D., Stegehuis, F., Majoor, M. J. & Luirink, J. (1993). A lipoprotein signal peptide plus a cysteine residue at the amino-terminal end of the periplasmic protein beta-lactamase is sufficient for its lipid modification, processing and membrane localization in *Escherichia coli*. *FEMS Microbiol Lett* **108**, 353–359.
- Rollof, J., Braconier, J. H., Soderstrom, C. & Nilsson-ehle, P. (1988). Interference of *Staphylococcus aureus* lipase with human granulocyte function. *Eur J Clin Microbiol Infect Dis* **7**, 505–510.
- Rose, J. M., Houston, C. W., Coppenhaver, D. H., Dixon, J. D. & Kurosky, A. (1989). Purification and chemical characterization of a cholera toxin-cross-reactive cytolytic enterotoxin produced by a human isolate of *Aeromonas hydrophila*. *Infect Immun* **57**, 1165–1169.
- 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. *Proc Natl Acad Sci USA* **74**, 5463–5467.
- Sankaran, K. & Wu, H. C. (1994). Lipid modification of bacterial prolipoprotein. *J Biol Chem* **269**, 19701–19706.
- Shine, J. & Dalgarno, L. (1974). The 3'-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to non-sense triplets and ribosome binding sites. *Proc Natl Acad Sci USA* **71**, 1342–1346.
- Spok, A., Stubenrauch, G., Schörgendorfer, K. & Schwab, H.

(1991). Molecular cloning and sequencing of a pectinesterase gene from *Pseudomonas solanacearum*. *J Gen Microbiol* **137**, 131–140.

Sugiura, M. (1984). Bacterial lipases. In *Lipases*, pp. 505–523. Edited by B. Borgstrom & H. L. Brockman. Amsterdam: Elsevier.

Thornton, J., Howard, S. P. & Buckley, J. T. (1988). Molecular cloning of a phospholipid-cholesterol acyltransferase from *Aeromonas hydrophila*: sequence homologies with lecithin-cholesterol acyltransferase and other lipases. *Biochim Biophys Acta* **959**, 153–159.

van Doorn, J., Oudega, B., Mool, F. R. & de Graaf, F. K. (1982). Subcellular localization of polypeptides involved in the biosynthesis of K88ab fimbriae. *FEMS Microbiol Lett* **13**, 99–104.

Vieira, J. & Messing, J. (1982). The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**, 259–268.

Wu, H. C. & Tokunaga, M. (1986). Biogenesis of lipoproteins in bacteria. *Curr Top Microbiol Immunol* **125**, 127–157.

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.

Received 23 July 1996; revised 10 October 1996; accepted 21 October 1996.