Molecular analysis and expression of the lipase of *Staphylococcus epidermidis*

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(Received 1 June 1992; revised 14 September 1992; accepted 8 October 1992)

Lipase of *Staphylococcus epidermidis* 9 was purified from culture supernatant fluid. Two polypeptides (51 and 43 kDa) were detected by SDS-PAGE, of which the 43 kDa polypeptide reacted with anti-lipase serum. The *S. epidermidis* 9 lipase gene (*gehC*) was cloned in *Escherichia coli* and localized to a 2.1 kb sequence by subcloning and transposon mutagenesis. The nucleotide sequence of *gehC* (2064 nucleotides) was determined and the predicted amino acid sequence of the encoded lipase (77 kDa) identified. A 97 kDa lipase was detected in extracts of *E. coli* harbouring *gehC* and in post-exponential-phase culture supernatant fluids of *S. epidermidis* 9. Data presented indicate that the lipase behaves anomalously during SDS-PAGE and that a pro-lipase is proteolytically processed in cultures of *S. epidermidis* 9 during growth.

Introduction

Staphylococcus epidermidis is the most prevalent and persistent *Staphylococcus* species on human skin and is present in large numbers over much of the skin area (Kloos, 1990). Staphylococci secrete extracellular enzymes which may have evolved as scavenging enzymes, degrading polymeric material (proteins, lipids, nucleic acids and polysaccharides) in the environment. These may provide a survival advantage by releasing low molecular mass compounds for growth (Arvidson, 1983).

Until recently, coagulase-negative staphylococci, such as *S. epidermidis*, were not considered to be important in causing disease and their presence in clinical specimens was considered to be due to contamination. In contrast, *Staphylococcus aureus* is an important pathogen causing a wide range of infections in man and other animals. As a result, attention has been directed to *S. aureus* and the coagulase-negative staphylococci have been studied much less. Recently, *S. epidermidis* has assumed greater importance in infections associated with implanted medical devices (Pulverer *et al.*, 1987). Coagulase-negative staphylococci are ideally situated to cause infections in such compromised hosts because of their

prevalence on the skin. The role of various extracellular enzymes in infections caused by *S. epidermidis* is unclear. Factors which have been implicated include α - and β -haemolysins, lipases and proteases, in addition to cell components which promote adherence to host cell surfaces (Baird-Parker, 1990).

The advent of molecular cloning methodology has facilitated the study of various staphylococcal gene products as putative virulence determinants. Mutants of *S. aureus*, deficient in α -haemolysin and protein A, have been constructed by allele replacement, allowing comparative studies with otherwise isogenic strains in animal models (O'Reilly *et al.*, 1986; Patel *et al.*, 1987).

As part of a programme to study the physiological and pathological role of *S. epidermidis* extracellular enzymes, lipase (EC 3.1.1.3) was chosen for study. Physiological studies comparing a lipase-deficient mutant with an otherwise isogenic strain would provide information concerning the role of the enzyme in staphylococcal nutrition and colonization. The initial stages of allele replacement, namely the cloning and expression of an *S. epidermidis* lipase gene (*gehC*) in *Escherichia coli* are described in this communication. The sequence of *gehC* and lipase expression in *S. epidermidis* cultures are also reported.

Methods

Bacterial strains, plasmids and phages. An isolate from the volar forearm, designated strain 9, was identified as *S. epidermidis* by the

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The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M95577.

Table 1. *Bacterial strains*

Strain	Genotype and relevant properties	Source or reference
C600	<i>lac thr leu thi tonA hsdR hsdM</i>	Appleyard (1954)
XAcSu ⁻	$\Delta lac-pro_{vIII} ara argE thi rif nal Su^-$; non-suppressing host for Tn5 mutagenesis with $\lambda 467$	Coleman & Foster (1981)
TG1	$\Delta lac pro supE thi hsdD5/F' traD36 proA^+ B^+ lacI^q \Delta lacZM15$	Wain-Hobson <i>et al.</i> (1985)
DS410	<i>minA minB rpsL</i> ; minicell producer	Dougan & Sherratt (1977)
XL-1 Blue	<i>RecA^- (recA1 lac endA gyrA96 thi hsdR17 supE44 relA1 {F^- proAB lacI^q lacZ\Delta M15 Tn10})</i>	Stratagene

Table 2. *Plasmids*

Plasmid	Markers	Other relevant properties	Reference
pBluescript	Ap ^r	Cloning vector	Stratagene
pBR322	Ap ^r Tc ^r	Cloning vector	Bolivar <i>et al.</i> (1977)
pIC20H	Ap ^r	Cloning vector	Marsh <i>et al.</i> (1984)
pUL5000	Ap ^r	2.5 + 1.2 kb <i>Hind</i> III fragments from $\lambda gehC$ cloned in pBR322	This study
pUL5001	Ap ^r	15.4 kb <i>Eco</i> RI fragment from $\lambda gehC$ cloned in pBR322	
pUL5003	Ap ^r	2.5 kb <i>Hind</i> III fragment from pUL5000 in pIC20H	
pUL5004	Ap ^r	1.2 kb <i>Hind</i> III fragment from pUL5000 in pIC20H	
pUL5005	Ap ^r	1.55 kb <i>Eco</i> RV fragment from pUL5000 in pIC20H	
pUL5007	Ap ^r	2.85 kb <i>Cla</i> I fragment from pUL5000 in pIC20H	
pUL5008	Ap ^r	pUL5007 with 2.85 kb <i>Cla</i> I fragment in opposite orientation	
pUL5009	Ap ^r	2.85 kb <i>Cla</i> I fragment from pUL5000 in pBluescript II KS	
pUL5010	Ap ^r	pUL5009 with 2.85 kb <i>Cla</i> I fragment in opposite orientation	This study
pUL5000 <i>gehC::Tn5</i>	Ap ^r Km ^r	Tn5 mutants deficient in lipase around colonies	

scheme of Kloos & Schleifer (1975). *E. coli* strains and plasmids used in this study are shown in Tables 1 and 2, respectively.

Bacteriological media. *E. coli* strains were grown in L broth and on L agar, and λ phages were propagated on λ base and top agar (Miller, 1972). *E. coli* DS410 was propagated in Brain Heart Infusion. For *S. epidermidis* 9 lipase production, modified forms of the synthetic medium of Taylor & Holland (1989) were used. The media were designated medium A and medium B (Table 3).

Enzyme assays. Acid phosphatase was assayed as described by Ingham *et al.* (1985). Proteinase was assayed as described by Ingham *et al.* (1985) except the reaction mixture comprised 1% (w/v) azocasein in 0.2 M-MOPS (pH 7.4) and the incubation time was 2 h. One unit of proteinase is defined as that amount which causes an increase in A_{440} of 0.001 h⁻¹. Lipase was assayed as described by Ingham *et al.* (1981) except an emulsion of 20% (w/v) triolein and 20% (w/v) gum arabic emulsified in 0.2 M-Tris/HCl (pH 7.5) was used as the substrate and the incubation time was 10 min. One unit of lipase activity is defined as that amount which releases 1 μ mol oleic acid min⁻¹.

Production of lipase. *S. epidermidis* was grown in 6 $\times$ 500 ml volumes of medium A in 2-litre baffles for 6 h with orbital shaking at

160 r.p.m. at 37 °C. Cultures were centrifuged (5200 g, 10 min) and the supernatant fluid was filtered through 0.2 μ m porosity filters. The filtrate was stored at 4 °C.

Purification of *S. epidermidis* lipase. All procedures were carried out at 0–4 °C.

(a) Ultrafiltration. Filtered culture supernatant fluid was concentrated by ultrafiltration with a PM10 Diaflo membrane (cut-off, 10 kDa) and desalted by diafiltration.

(b) Anion-exchange chromatography. Concentrated lipase was applied to a DEAE-Sephacrose column (1.6 $\times$ 22 cm) pre-equilibrated with 50 mM-Tris/HCl (pH 7.8). Fractions were eluted with 100 ml 50 mM-Tris/HCl (pH 7.8) followed by a linear salt gradient (0–1.5 M-NaCl, 300 ml) at a flow rate of 24 ml h⁻¹. Fractions with a constant specific lipase activity were pooled and lyophilized.

(c) Gel-filtration chromatography. Lyophilized lipase was reconstituted in distilled water and applied to a Sephadex G-100 column (1.6 $\times$ 25 cm) pre-equilibrated with 50 mM-Tris/HCl (pH 7.0). Fractions were eluted at a flow rate of 6.3 ml h⁻¹ in the same buffer. The column was calibrated with Blue Dextran 2000, bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12.4 kDa) (Sigma). Fractions from the Sephadex G-100 column with the highest specific activity were pooled and applied to a Sephadex G-25 column

Table 3. Composition of synthetic media

Component	Final concn (g l ⁻¹)	
	Medium A	Medium B
L-Alanine	2.40	9.60 × 10 ⁻¹
L-Arginine	3.6 × 10 ⁻¹	1.44 × 10 ⁻¹
L-Aspartic acid	2.40	9.60 × 10 ⁻¹
L-Cystine	2.4 × 10 ⁻¹	9.6 × 10 ⁻²
L-Glutamic acid	2.40	9.60 × 10 ⁻¹
Glycine	2.40	9.60 × 10 ⁻¹
L-Histidine	4.8 × 10 ⁻¹	1.92 × 10 ⁻¹
L-Isoleucine	6.0 × 10 ⁻¹	2.40 × 10 ⁻¹
L-Leucine	6.0 × 10 ⁻¹	2.40 × 10 ⁻¹
L-Lysine	6.0 × 10 ⁻¹	2.40 × 10 ⁻¹
L-Methionine	1.8 × 10 ⁻¹	7.2 × 10 ⁻²
L-Phenylalanine	2.0 × 10 ⁻¹	8.0 × 10 ⁻²
L-Proline	2.40	9.60 × 10 ⁻¹
L-Serine	2.40	9.60 × 10 ⁻¹
L-Threonine	2.40	9.60 × 10 ⁻¹
L-Tryptophan	6 × 10 ⁻²	2.4 × 10 ⁻²
L-Tyrosine	1.8 × 10 ⁻¹	7.2 × 10 ⁻²
L-Valine	4.8 × 10 ⁻¹	1.92 × 10 ⁻¹
Nicotinic acid	2.0 × 10 ⁻²	2.0 × 10 ⁻²
Biotin	6 × 10 ⁻⁵	6 × 10 ⁻⁵
Thiamin. HCl	9.0 × 10 ⁻³	9.0 × 10 ⁻³
MgSO ₄ · 7H ₂ O	2.5 × 10 ⁻¹	4.93 × 10 ⁻²
ZnCl ₂	3.4 × 10 ⁻³	6.8 × 10 ⁻⁴
CuSO ₄ · 5H ₂ O	5 × 10 ⁻⁴	1.0 × 10 ⁻⁴
MnSO ₄ · 4H ₂ O	5.6 × 10 ⁻³	1.12 × 10 ⁻³
NaCl	5.8 × 10 ⁻³	1.16 × 10 ⁻³
CaCl ₂ · 2H ₂ O	5.0 × 10 ⁻³	1.00 × 10 ⁻³
CoCl ₂ · 6H ₂ O	2.4 × 10 ⁻³	4.8 × 10 ⁻⁴
FeCl ₃ · 6H ₂ O	2.7 × 10 ⁻²	5.4 × 10 ⁻⁴
KH ₂ PO ₄	1.34	1.34
Na ₂ HPO ₄	5.70	5.70
(NH ₄) ₂ SO ₄	5.00	2.00
Glucose	4.00	2.00

(1 × 13 cm). Fractions were eluted at a flow rate of 14.4 ml h⁻¹ in distilled water.

(d) Cation-exchange chromatography. Lipase was applied to a CM-Sepharose column (1 × 10 cm) pre-equilibrated with 50 mM-sodium phosphate buffer (pH 7.0). Fractions were eluted in the same buffer at a flow rate of 30 ml h⁻¹ and, after 20 ml had eluted, a linear salt gradient was applied (0–0.5 M-NaCl, 40 ml). Lipolytic fractions were stored at –20 °C.

Protein assay. Elution of protein from chromatography columns was monitored at 280 nm. Protein concentration was determined by the method of Bradford (1976), using bovine serum albumin as the standard.

SDS-PAGE. SDS-PAGE was performed using 7.5% (w/v) gels as described by Laemmli (1970). The molecular mass standards (Sigma) were carbonic anhydrase (29 kDa), egg albumin (45 kDa), bovine albumin (66 kDa), phosphorylase b (97.4 kDa), β-galactosidase (116 kDa) and myosin (205 kDa). Proteins were visualized using the GELCODE Silver Staining System (Pierce). Western immunoblotting was carried out with the Pharmacia Novablot apparatus as described in the manual. After electrophoresis, proteins were transferred to nitrocellulose and blots were developed as described by Russell *et al.* (1985).

Antisera. Antiserum was raised against purified lipase from the cation-exchange column. Lipase doses (50 µg) were administered to New Zealand White rabbits using the multiple inoculation regime of

Ingham *et al.* (1984). The antiserum was absorbed with a concentrated extract of *E. coli* C600 before use. Horseradish-peroxidase-conjugated swine anti-rabbit globulins were from Dakopatts.

DNA manipulations. Restriction endonucleases and other enzymes were obtained from Pharmacia or Gibco/BRL and used according to the manufacturers' instructions. A library of *S. epidermidis* 9 genomic DNA was constructed in the replacement vector, λL47.1, as described by O'Toole & Foster (1986), except that genomic DNA was partially cleaved with *Mbo*I, and λL47.1 was digested with *Bam*HI. DNA manipulations were performed using standard procedures (Maniatis *et al.*, 1982).

The 2.85 kb fragment of pUL5000 was subcloned into pBluescript II (KS) in both orientations, forming pUL5009 and pUL5010, and deletions were generated with exonuclease III and mung bean nuclease. The nucleotide sequence was determined by the dideoxynucleotide method of Sanger *et al.* (1977) using [³⁵S]dATP and T7 DNA polymerase.

Screening of recombinant phages for lipase production. Recombinant phages were plated on *E. coli* C600 and plaques were overlaid with 7 ml tributyrin agar, comprising 3 vols 1% (w/v) Agar no. 1 (Oxoid) in 50 mM-Tris/HCl (pH 7.5) and 1 vol. tributyrin emulsion (4%, v/v, tributyrin and 0.4%, w/v, polyvinyl alcohol emulsified in 50 mM-Tris/HCl, pH 7.5). After 18 h incubation at 37 °C, recombinant phages from presumptive lipolytic plaques (zone of clearing above plaques) were purified, plated on *E. coli* C600 and overlaid with 5 ml triolein agar comprising 20 vols Blood Agar Base (Oxoid), containing 30 µg·ml⁻¹ Victoria Blue B (Difco), and 1 vol. triolein emulsion (20%, w/v, triolein and 4%, w/v, gum arabic emulsified in distilled water). Lipase-expressing recombinants were indicated by a blue zone above plaques after 24 h at 37 °C.

Screening of colonies for lipase production. Tributyrin agar comprised 24 vols 2TY [1%, w/v, Tryptone; 1%, w/v, Yeast Extract; 1%, w/v, Agar no. 1 (components from Oxoid) and 0.5%, w/v, NaCl] and 1 vol. tributyrin emulsion (20%, v/v, tributyrin and 2%, w/v, polyvinyl alcohol emulsified in distilled water). Triolein agar was prepared as described for screening phages. Lipolytic activity was indicated by a zone of clearing and a zone of blue precipitation around colonies on tributyrin and triolein agars, respectively, after 24 h at 37 °C.

Plaque immunoblotting. Lipase-expressing λ plaques were detected as described by Russell *et al.* (1985). The nitrocellulose was sequentially incubated with anti-lipase serum and peroxidase-conjugated swine anti-rabbit immunoglobulins.

Immunochemical analysis of lipase. Extracts of plasmid-carrying strains were prepared by osmotic shock, as described by Neu & Heppel (1965). Proteins were separated by SDS-PAGE, subjected to Western blotting and blots were developed as described for plaque immunoblotting.

Transposon mutagenesis. Transposon Tn5 insertion mutagenesis was performed by infecting *E. coli* XAcSu⁻ carrying pUL5000 with λ467 as described previously (Coleman & Foster, 1981). Lipase-negative mutants were detected on tributyrin and triolein agars containing ampicillin (100 µg ml⁻¹) and kanamycin (50 µg ml⁻¹).

Nucleotide sequence analysis. Sequence alignments were performed using the multiple sequence alignment CLUSTAL 4 (Higgins & Sharp, 1989), on an IBM AT-compatible microcomputer.

Minicells. *E. coli* DS410 was transformed with plasmids, and minicells were prepared and labelled as described by Dougan & Sherratt (1977). Plasmid-encoded polypeptides were analysed by SDS-PAGE and autoradiography using ¹⁴C-labelled molecular mass standards (Amersham).

Fig. 1. Growth of, and extracellular product formation by, *S. epidermidis* 9 in (a) medium A and (b) medium B. ●, $\ln OD_{600}$; ○, pH; ▲, proteinase; □, lipase; ■, extracellular protein.

Results

Purification of *S. epidermidis* lipase

A defined synthetic medium (medium A) was employed as the growth medium for the production of *S. epidermidis* 9 lipase for purification. Maximum specific lipase activity [$14 \text{ U (mg protein)}^{-1}$] was produced after 6 h incubation (Fig. 1a). Culture supernatant fluid was filtered and concentrated 18-fold by ultrafiltration. Subsequent chromatography of this material on DEAE-Sephacel resulted in two peaks of lipase activity, with 93% eluting without binding to the column. Proteinase and acid phosphatase bound to the column and eluted with 0.84 and 0.73 M-NaCl, respectively. Fractions with highest specific activity of unbound lipase were combined and subjected to Sephadex G-100 chromatography. Lipase eluted as a broad peak of activity, with an apparent molecular mass of 60–200 kDa. Fractions with highest specific lipase activity (corresponding to 80 kDa) were combined, desalted and applied to a CM-Sephacel column. The lipase bound to the cation-exchange and eluted with 0.17 M-NaCl. The final purification factor was ninefold with a 1% yield.

Two polypeptides, of 43 and 51 kDa, were observed when the material from the cation-exchange column was analysed by SDS-PAGE and silver staining. Only the 43 kDa polypeptide was detected when the material was analysed by Western immunoblotting with anti-lipase serum.

Cloning of lipase gene in *E. coli*

A method, employing the substrates tributyrin and triolein, was developed for detecting lipase-expressing recombinant phages. Tributyrin was used as the primary screen due to its high sensitivity to lipase activity, although it is nonspecific. Triolein was used as a confirmatory screen as it is specific for lipase. Recombinant phages from the *S. epidermidis* gene library ($1.4 \times 10^7 \text{ p.f.u. ml}^{-1}$) were screened for lipase activity by overlaying plaques with tributyrin agar. Zones of lipolysis were observed above 0.1% of the plaques after overnight incubation. Lipase activity was confirmed using triolein agar, and by plaque immunoblotting with anti-lipase serum. One of the phages, designated λgehC , was chosen for further study.

Subcloning of *gehC*

The *Hind*III fragments of λgehC were subcloned into the *Hind*III site of pBR322 in *E. coli* C600 and lipolytic transformants were detected on tributyrin and triolein agars. Analysis of plasmid DNA revealed two *Hind*III fragments (2.5 and 1.2 kb) which were contiguous in λgehC (Fig. 2). One of the transformants was selected for further study and the plasmid designated pUL5000. Lipase activity was detected in concentrated culture supernatant fluids and periplasmic extracts of *E. coli* C600 harbouring pUL5000 [0.13 and $0.12 \text{ U (mg$

Fig. 2. Map of the cloned lipase gene. The thin line represents cloned *S. epidermidis* 9 sequences in *λ*gehC and pUL5000. The triangles show the location of Tn5 insertions. Restriction endonuclease cleavage sites are abbreviated as follows: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; E_v, *Eco*RV; H, *Hind*III; M, *Mbo*I; P, *Pvu*II; S, *Sph*I. Only the *Eco*RI and *Hind*III sites were mapped in *λ*gehC.

protein)⁻¹, respectively]. Lipase activity was not detected in equivalent samples from *E. coli* harbouring pBR322.

To further localize *gehC* in pUL5000, a series of subclones was constructed. The 2.85 kb *Cla*I fragment from pUL5000 was subcloned into the *Cla*I site of pIC20H in *E. coli* TG1, and plasmids with the insert in both orientations were identified (pUL5007 and pUL5008). High levels of lipase were expressed by *E. coli* TG1 harbouring pUL5008 in the presence of IPTG, whereas lower levels were produced in the presence of glucose and absence of IPTG, as detected by the colony assay. Low levels of lipase were also expressed from pUL5007 and expression was not affected by IPTG or glucose. These results suggest that both the *lac* and *gehC* promoters contribute to expression of lipase from pUL5008, whereas expression from pUL5007 is dependent only on the *gehC* promoter.

To localize *gehC* more precisely, twelve independent Tn5 insertion mutations which prevented the expression of lipase from pUL5000 were mapped. The mutations covered 2.1 kb and spanned the central *Hind*III fragment (Fig. 2).

To confirm the Tn5 insertion mutagenesis data, the 2.5 kb *Hind*III fragment was cloned into the *Hind*III site of pIC20H in *E. coli* TG1. Lipase was expressed only when the fragment was in one orientation (pUL5003). The 1.2 kb *Hind*III and 1.55 kb *Eco*RV fragments of pUL5000 were ligated to *Hind*III- and *Eco*RV-cleaved pIC20H, respectively, forming pUL5004 and pUL5005. These plasmids did not express lipase activity in *E. coli* TG1.

Nucleotide sequencing

The nucleotide sequence of the 2.85 kb *Cla*I fragment encoding *gehC* was determined in both orientations. A single open reading frame of 2064 nucleotides which

encodes a protein of 688 amino acid residues with a predicted molecular mass of 77 kDa was identified (Fig. 3). The Tn5 insertion mutations are located in this open reading frame. It is likely that the GTG codon at position 121–123 nucleotides is the translational start site, even though GTG is used very rarely, because a potential ribosome-binding site (AGAGGTG) is located 7 nucleotides upstream and the predicted amino acid sequence of the resulting protein shows homology with other staphylococcal lipases (Fig. 4) (Gotz *et al.*, 1985; Lee & Iandolo, 1986). The locations of putative –35 (TTGGAT) and –10 regions (TAATAT) are shown in Fig. 3.

The pattern of codon usage is similar to other staphylococcal genes, with a strong bias towards A and U nucleotides in the wobble positions (Gotz *et al.*, 1985; Phonimdaeng *et al.*, 1990).

The hydropathy profile of the predicted N-terminal amino acid sequence indicates the presence of a signal sequence. A short hydrophilic sequence (2–12 residues) is followed by a longer hydrophobic sequence (13–31 residues) and ends in Ala-Gln-Ala, an expected sequence preceding the cleavage site for a signal peptidase (Perlman & Halvorson, 1983).

Comparison of *gehC* with other lipases

The aligned amino acid sequences of the *S. epidermidis*, *S. hyicus* and *S. aureus* lipases are shown in Fig. 4. The N-terminal regions are distantly related, as indicated by the predominance of conserved residues. The C-terminal regions, arbitrarily starting at the lysine residue indicated in Fig. 4, are closely related, with 43% of the 386 residues being identical and 35% being conservative changes.

A nine-residue segment containing some hydrophobic residues and the consensus sequence Gly-X₁-Ser-X₂-Gly, where X₁ is Tyr or His, has been identified in all known microbial and mammalian lipase sequences. The location of this segment in the sequence of *S. epidermidis* lipase is shown in Fig. 4. The serine residues in the *Mucor miehei* and human pancreatic lipases are part of a Ser..His..Asp catalytic triad structurally related to trypsin (Brady *et al.*, 1990; Winkler *et al.*, 1990). It will be interesting to note whether the staphylococcal lipases are similar in this respect.

Analysis of *gehC*-encoded proteins in *E. coli*

Periplasmic extracts of *E. coli* harbouring pUL5000 and pUL5008 contained an immunoreactive protein of 97 kDa which was not detected in extracts of cells harbouring pBR322, pIC20H or pUL5000*gehC*::Tn5

CAAACCCATTTTCTAGTAAATTTTGGATTATACCACCTATAATTATTAATATTTTCATCTAACAAATTATCGA	75
-35 -10	
GAAAAATTCATTTTAAATAAACAAATAGATAGAGGTGCTAATTGTGAAGACAAGACAAAACAAATACAGTATT	150
SD M K T R Q N K Y S I	10
CGTAAATTTAGCGTAGGTGCATCATCCATTCTGATTGCAGCATTATTATTTATGGGTGGAGGATCAGCTCAGGCA	225
R K F S V G A S S I L I A A L L F M G G G S A Q A	35
PvuII	
GCTGAGCAACAACAGGATAAGCGAACTGTTGAAAAATAGCACACACAATCTATTGGGGATGGAATGAAAAGTTA	300
A E Q Q Q D K G T V E N S T T Q S I G D G N E K L	60
HindIII	
AGTGAACAACAATCAACGCAAAATAAGAATGTTAATGAGAAAAGTAATGTTAATTCTATTACTGAAAATGAAAGC	375
S E Q Q S T Q N K N V N E K S N V N S I T E N E S	85
TTACATAACGAAACACCAAAAAACGAGGATTTGATTTCAGCAACAAAAAGATTCTAAAATGACAATAAATCTGAA	450
L H N E T P K N E D L I Q Q Q K D S Q N D N K S E	110
TCTGTACTTGAACAAAAATAAGAGAATCGAGCATTGTTCAAAATCATTCCGAAGAGAAACCACAACAAGAACAA	525
S V V E Q N K E N G A F V Q N H S E E K P Q Q E Q	135
SphI	
GTTGAAGTGAAGGATGCTAGTGAACAAATCAAATTTTACTCTAAAAGCAGCACAGTCCAATGAAGATGTG	600
V E L E K H A S E N N Q T L H S K A A Q S N E D V	160
PvuII	
AAAATAAACCTTCACAACCTCGATAATACAGCTGCCAACAAAGAGACTCTAAAAAGAGAATTTGAGTAAACAA	675
K T K P S Q L D N T A A K Q E D S Q K E N L S K Q	185
GATACACAATCATCTAAACTACTGATTTACTACGAGCAACAGCTCAAAATCAATCAAAAGATAGCCAATCAACA	750
D T Q S S K T T D L L R A T A Q N Q S K D S Q S T	210
CAAGACATAAATAAACAAGTAAATAACGACACTCAACAAGTGACTGCTAAGAACGATGACCCCAAAGTTGAATCA	825
E E I N K E V N N D T Q Q V T A K N D D A K V E S	235
TTTAATTTAAATAGTAAAGAGGAACCCCTTAAAGTTGACAAGCAACCGAATCCAATACAGATAAAGATAAATCT	900
F N L N S K E E P L K V D K Q A N P T T D K D K S	260
TCTAAAAATGATAAAGGCTCTCAAGATGGTCTCGCTAATTTAGAAAGTAATGCTGTTGCTACAATAAACAACAG	975
S K N D K G S Q D G L A N L E S N A V A T T N K Q	285
TCTAACCAACAAGTCACTGAAAAAATGAGGATCAAAACAAATAAATCAGCAAAACAAAAACAATATAAAAAATAAT	1050
S K Q Q V S E K N E D Q T N K S A K Q K Q Y K N N	310
EcoRV	
GATCCAATTATTTTCTAGTACATGGTTTCAATGGATTACAGACGATATCAACCCATCAGTGCTAACGCATTATTGG	1125
D P I I L V H G F N G F T D D I N P S V L T H Y W	335
GGTGGCGATAAAATGAATATTGCGCAAGATTTGGAAGAAAATGGATATGAGGCTTATGAAGCAAGTATAAGTGCA	1200
G G D K M N I R Q D L E E N G Y E A Y E A S I S A	360
TTTGGTAGTAATATGACCGTGCTGTTGAGTTATACTACTACATCAAAGGTGACCGTGTGACTATGGTGCAGCA	1275
F G S N Y D R A V E L Y Y Y I K G G R V D Y G A A	385
CACGCAGCTAAATATGGTCATGAGCGTTACGGTAAACCTATGAAGGTGTTTATAAAGATTGGAACCAAGGTCAA	1350
H A A K Y G H E R Y G K T Y E G V Y K D W K P G Q	410
AAAATACATTTAGTTGGTCATAGTATGGGTGGTCAAACAATTCGTCAATTAGAAGAGCTATTGAGACATGGTAAT	1425
K I H L V G H S M G G Q T I R Q L E E L L R H G N	435
CCAGAAGAAGTTGAATATCAAAAACAACATGGTGGGAAATTTCTCCATTATACCAAGGTGGCCACGACAATATG	1500
P E E V E Y Q K Q H G G E I S P L Y Q G G H D N M	460
GTGTCATCTATTACAACACTCGGTACACCACATAATGGTACACATGCGTCAGACTTATTAGCTAACGAACCGATT	1575
V S S I T T L G T P H N G T H A S D L L G N E A I	485
GTACCCCAACTTGCATATGACGTAGGTAAATGTATGGTAATAAAGATTACGTGTAGACTTTGGGTTAGAACAC	1650
V R Q L A Y D V G K M Y G N K D S R V D F G L E H	510

TGGCGATTAAACAAAAACCAACGAATCATATATTCAATATGTTAAACGTGTTCAAAATTCAAAACGTGGAAA	1725
W G L K Q K P N E S Y I Q Y V K R V Q N S K L W K	535
TCAAAAGATAGTGGTTTACACGATTTAACACGGCATGGCGCAACAGATTTAAACCGAAAAACATCATTAAATCCT	1800
S K D S G L H D L T R D G A T D L N R K T S L N P	560
AATATTGTATATAAACTTATACTGGCGAGTCAACACATAAAACATTGGCAGGAAAAACAAAAGCTGATCTTAAC	1875
N I V Y K T Y T G E S T H K T L A G K Q K A D L N	585
ATGTTCTTACCATTACAATTACTGCTAATTTAATTGGAAAAGCTAAAGAGAAAGAATGGAGAGAAAATGATCGA	1950
M F L P F T I T G N L I G K A K E K E W R E N D G	610
CTTGTTTCAGTCATTTCTTACAACATCCATTTAATCAAAAATATGTTGAAGCTACAGATAAAAATCAAAAAGGT	2025
L V S V I S S Q H P F N Q K Y V E A T D K N Q K G	635
GTATGCCAAGTAACTCCAACAAAACATGACTGGCATCATGTAGACTTTGTAGGCCAAGACACTACAGATACAAAA	2100
V W Q V T P T K H D W D H V D F V G Q D S T D T K	660
CGTACTACAGATGAATTGCAACAGTTCTGGCATGGTCTTGCTGAAGATTTAGTACAAAAGTGAACAATTAACATCA	2175
R T R D E L Q Q F W H G L A E D L V Q S E Q L T S	685
ACAAATAAATAAATGATATAAGGTATAAACCTAACCTAATTTTATATAGATAGGAGTGAAGATACATGACAGGTG	2250
T N K *	688

Fig. 3. Nucleotide sequence of the *S. epidermidis* 9 *gehC* gene and the deduced amino acid sequence of lipase. Putative -35, -10 and ribosome-binding site (SD) sequences are underlined (thin line). Restriction sites for *PvuII*, *HindIII*, *SphI* and *EcoRV* are indicated. The amino acid sequence obtained by sequencing the N-terminus of the 43 kDa lipase is underlined (thick line).

plasmids. A polypeptide of the same molecular mass was expressed, in addition to *bla*-encoded polypeptides, in *E. coli* minicells harbouring pUL5000 and pUL5008 (Fig. 5). The 97 kDa polypeptide was not detected in extracts from *E. coli* minicells harbouring pBR322 or pUL-5000*gehC*:Tn5 plasmids.

Expression of lipase in *S. epidermidis*

Preliminary data indicated that a 97 kDa lipase was produced by *S. epidermidis* 9 when medium B was used as the growth medium.

The growth kinetics and extracellular levels of lipase, proteinase and protein produced by *S. epidermidis* 9 in medium A and medium B are shown in Fig. 1. Lipase activity was detected in culture supernatant fluids during the post-exponential growth phase in both media.

In medium A, lipase activity increased to a maximum after 6 h incubation and declined thereafter. This was associated with an increase in proteolytic activity. The major polypeptides detected in Western immunoblots of supernatant fluids from cultures after 3–4 h incubation were 68, 64 and 46 kDa. The 68 and 64 kDa proteins were not detected after 4 h incubation. The levels of an additional polypeptide (43 kDa) increased throughout growth to a maximum after 8 h incubation (Fig. 6).

In medium B, lipase activity remained relatively constant after reaching a maximum after 6 h incubation. Proteins of 97, 64 and 46 kDa were detected in culture supernatant fluids by Western immunoblotting (Fig. 6) and proteolytic activity was not detected.

Identification of *S. epidermidis* lipase

In addition to the observation that antiserum which neutralized lipase activity reacted with the 43 kDa polypeptide in Western immunoblotting, the latter was identified as lipase by N-terminal amino acid sequencing. The location of the N-terminus of the 43 kDa lipase in relation to *gehC* is shown in Fig. 3. The predicted molecular mass of the lipase is 43.9 kDa, which agrees with that determined by SDS-PAGE. The N-terminal amino acid sequence of the 51 kDa polypeptide (AIDD-LTKVLNYTK) shows no homology to the predicted amino acid sequence of *S. epidermidis* lipase.

Discussion

In a defined synthetic medium, *S. epidermidis* 9 produced lipase, proteinase and acid phosphatase activities and more than 50 proteins were detected by SDS-PAGE. After purification, only lipase activity and two polypeptides were detected by SDS-PAGE and silver staining.

Purification of the lipase for antiserum production presented difficulties due to the nature of the lipase. Lipase species with molecular masses in the range 60–200 kDa were observed during gel-filtration and this was probably due to aggregation. Lipases from several sources have been shown to aggregate under certain conditions, forming high molecular mass forms (Kimura *et al.*, 1972; Vadehra, 1974; Ingham *et al.*, 1981), including an *S. aureus* lipase with a molecular mass of 43 kDa by SDS-PAGE which aggregated to a 300 kDa

H	1	MKETKHQHTFSIRKSAYGAASVMVASCFVIGGGVAEAND-----STTQTTTPLEVAQTS	55
A	1	MLRGQEERKYSIRKYSIGVSVLAAT-MFVVSSEAQASEKTSTNAAQKETLNQPGEQC	59
E	1	M--KTRQNKYSIRKFSVGASSILIAA-LLFMCGGSAQAAEQQDKCTVENSTTQSIGDGN	57
		* . . . . . **** . * . * . * . . . . * . * . . . . *	
H	56	QQETHHTQTP---VTSLHTATPEHVDSDKEATPL-PEKAESPKEVTVQP-----	101
A	60	NAITSHQNQSGCKQLDDMHKENGSGTVTEGKDTLQSSKHQSTQNSKTIRTONDNQVKQDS	119
E	58	EKLSEQSTQNKVN---EKSNNVSI TE-NESLH---NETPKNEDLIQQQKDSQNDNKS	109
		. . . . *	
H	102	----SSHTQEVPAHKKTKQQPAYKDKTVPESTIASKSVESNKATENEMSPVEHHASNYE	157
A	120	ERQGSQKSH-----QNNATNTERKNDQVQNTTHAERNGSQSTTSQSNVDKQSQPSIPAQ	174
E	110	ESVVEQNKGCAFVQNHSEKPPQEQVELEKHAENNTLHSAQAQSNEDVKTKPS-QLD	168
		. . . .	
H	158	K---REDRLNETTPPSVDREFSH--KIINNTHVNPKTDCQTNVNVDTKTI DT-----	206
A	175	KVIPNHDKAAPSTTPPSNDKTAPKSTKAQDATTDKHPNQ-QDTHQPAHQI I DAKQDDTV	233
E	169	NTAAKQEDSQKENL--SKQDTQSSKTTDLLRATAQNSKDSQSTEEINKEVNDTQQVTA	226
		. . . . * . . . . . * . . . . . *	
H	207	-----VSPKDDRIDTAQPKQVDVPKENTTAQNKFTSQA---SDKKPTVKAAPKA	252
A	234	RQSEQKPVQVGLSKH-----IDGQNSPEKPTDKNTDNKQLIKDALQAPKTRSTTNAADA	288
E	227	KNDDAKVESFNLNSKEEPLKVDKQANPTTDDKDKSSKNDKGSQDGLANLESNAVATTNKQS	286
		. . . .	
H	253	-----VQNPENPKNDPFVVFHGFVGEVAAKG-ENHWGGTKANLRNHL	297
A	289	KKVRPLK----ANQVQPLNKY----PVVFHGFGLVGDNAPALYPNYWGNKFKVIEEL	340
E	287	KQVSEKNEDQTNKSAKQKQYKNDPI I LVHGFNGFTDDINPSVLTHYWGDKMNI RQDL	346
		. ↑ . . . . . * . . **** * . . . . **** * . . . . *	
H	298	RKAGYETYEASVSALASNHERAVELYYLKGGRVGYAAHSEKYCHERYGKTYEGVLKDW	357
A	341	RKGCYNVHQASVSAFGSNYDRAVELYYYIKGCRVDYGAHAHAAYCHERYGKTYGIMPVW	400
E	347	EENGYEAYEASISAFGSNYDRAVELYYYIKGCRVDYGAHAHAAYCHERYGKTYEGVYKDW	406
		. . ** . . . . ** . . . . . ***** . ***** . ***** . * . . *	
H	358	KPGHPVHFIGHSMGGQTI RLLEHYLRFGDKAEIAYQQQHGGI I SELFKGQDNMVTSI TT	417
A	401	EPGKKVHLVGHSMGGQTI RLMEFLRNGNKEE IAYHKAHGEI SPLFTGCHNNMVSI TT	460
E	407	KPGQKIHLVGHSMGGQTI RLLEELLRHGNPEEVEYQKHGGEI SPLYQCGHNNMVSI TT	466
		. ** . * . ***** . * . ** * . * . . . . ** * . * . . **** * . **	
H	418	IATPHNCTHASDDIGNTPTIRN ILYSFAQM-SSHLGTIDFGMDHWGFKRKDGESLTDYNK	476
A	461	LATPHNGSQAADKFCNTEAVRKIMFALNRFMGNKYSNIDLGLTQWGFQKLPNESYIDYIK	520
E	467	LGTPHNCTHASDLLGNEAIVRQLAYDVGMKYNKDSRVDFGLEHWGLKQKPNESYIQYVK	526
		. ***** . * . * . . . . * . . . . ** . * . . * . *	
H	477	RIAESKIWDSEDTGLYDLTREGAEKINQKTELNPNIYKTYTGVAETHETQLCKHIADLGM	536
A	521	RVSKSKIWTSDDNAAYDLTLDCSAKLNNMTSMNPNIYTTYTGVSSTGCLGYENPDLGT	580
E	527	RVQNSKLWKSCKDGLHDLTRDGCATDLNRKTSLNPNIVYKTYTGESTHKTACKQKADLNM	586
		* . ** . * . * . . . . . *** . * . . . . . ***** * . ***** . * . . *	
H	537	EFTKI LTGN YIGSVDDI LWRPN DGLVSEISSQHPSDEKNI SV-DENSELHKGTWQVMPTM	595
A	581	FFLMATTSRIIGHDAREWRKNDGVVPISSLHPSNQPFVNVNDEPATRRGIWQVKPII	640
E	587	FLPFTITCNLIGKAKEKEWRENDGLVSVISSQHPFNQKYVEATDKN---QKGVWQVTPTK	644
		. . . . . * . . ** . . . . . ** * . * . * . * . . . . * . * . *	
H	596	KGWDHSDFI GNDALDTKHSALIELTNFYHSISDYLMRIEKAEST----KNA	641
A	641	QGWHDVDFIGVDFLDFKRGKGAELANFYTG IINDLLRVEATESKGTQLKAS	690
E	644	HDWDHVDVFGQDSTDTKRTDELQFQWHCLAEDLVQSEQLTSTNK-----	688
		. . **** ** * . * . * . . . . . * . . . . * . . . . * . *	

Fig. 4. Alignments of amino acid sequences of lipases from: H, *S. hyicus* subsp. *hyicus* DSM 20459 (Gotz *et al.*, 1985); A, *S. aureus* PS54 (Lee & Iandolo, 1985); and E, *S. epidermidis* 9 (this study). Sequences were derived from DNA sequences and each contains a putative signal sequence. The amino acid sequences were aligned using the multiple alignment program CLUSTAL 4 (Higgins & Sharp, 1989). *, Identical residues; ., conservative amino acid replacements; ↑, lysine residue dividing N- and C-terminal regions. The consensus sequence containing a serine residue is underlined.

Fig. 5. Autoradiograph of plasmid-encoded polypeptides in *E. coli* minicells. Track 1, pBR322; track 2, pUL5000; tracks 3 and 4, pUL5000*gehC*::Tn5.

Fig. 6. Immunoblot of proteins (6 µg per track) in culture supernatant fluids of *S. epidermidis* 9 at various culture incubation times. Tracks 1, 2 and 3 (medium A) 4, 6 and 8 h incubation, respectively; tracks 4 and 5 (medium B) 5 and 9 h incubation, respectively.

form during gel-filtration (Tyski *et al.*, 1983). The situation was further complicated as a result of the partial degradation of the lipase by *S. epidermidis* proteinase(s). The latter problem was only apparent after lipase purification had been achieved, and antiserum had been raised and used in Western blotting.

Results suggest that it is possible to purify different molecular mass forms of *S. epidermidis* lipase depending on the lipase species in the culture supernatant fluid. In turn, this will depend on the incubation time at which

cultures are harvested during batch culture, and levels of proteolytic activity. The presence of several molecular mass forms of lipase would also influence the lipase yield and specific activity resulting from purification. In the present study, a 43 kDa lipase was purified from *S. epidermidis* 9 culture supernatant fluid. Lipases produced by other staphylococcal species are of a similar molecular mass (43–46 kDa) (Jurgens *et al.*, 1981; Tyski *et al.*, 1983; Van Oort *et al.*, 1989). The nature of the 51 kDa protein which co-purified with the lipase is unclear and requires further investigation.

The *S. epidermidis* 9 lipase gene (*gehC*) was cloned in λL47.1 and expressed in *E. coli*. The gene was subcloned in pUL5000, localized by transposon Tn5 mutagenesis and the nucleotide sequence determined. From the Tn5 mutagenesis data, it appeared unlikely that the 2.5 kb *Hind*III fragment in pUL5000 would be sufficient to encode lipase activity because the 5' end of *gehC* is located on the adjacent 1.2 kb *Hind*III fragment (Fig. 2). Lipase activity was detected, however, when the 2.5 kb *Hind*III fragment was cloned in pIC20H in one orientation, forming pUL5003. Examination of the nucleotide sequence of *gehC* indicated that an in-frame translational fusion would occur between *lacZ* and *gehC*. It is likely, therefore, that the lipase expressed from pUL5003 was a fusion protein formed between β-galactosidase and lipase. Lipase activity was not detected when the 1.2 kb *Hind*III and 1.55 kb *Eco*RV fragments were subcloned in either orientation in pIC20H. This was not surprising because these fragments do not contain the serine residue proposed to participate in either substrate binding or catalytic activity of lipases (Brady *et al.*, 1990; Winkler *et al.*, 1990).

The molecular mass of the lipase encoded by *gehC*, as predicted from the nucleotide sequence, was 77 kDa. Evidence indicating that the 97 kDa protein is the *gehC* translation product and that the 77 kDa lipase migrates anomalously on SDS-PAGE gels include the following: (1) *E. coli* minicells contained a single protein of 97 kDa in addition to vector-encoded polypeptides and this reacted with anti-lipase serum; (2) insertion of Tn5 within the *gehC* open reading frame abolished both lipase activity and the 97 kDa protein in *E. coli* minicells; and (3) lipolytic extracts of *E. coli* containing pUL5000 or pUL5008 contained an immunoreactive protein of 97 kDa, which was absent in Tn5 mutants and in vector-containing strains. Furthermore, it is likely that the anomaly occurs at the N-terminal region of the lipase because the molecular mass of the processed 43 kDa lipase, as determined by SDS-PAGE, is the same as that predicted from the nucleotide sequence downstream of the cleavage site.

In addition to small polypeptides, the 97 kDa protein was produced in lipolytic *S. epidermidis* 9 cultures (in

medium B) in which no extracellular proteinase was detected. In cultures with detectable lipase and proteinase activities (in medium A), not only was the lipase activity reduced after proteinase was produced (after 6 h incubation), but the 97 kDa polypeptide was not detected by Western blotting. In this case, immunoreactive polypeptides were in the range 68–43 kDa. The results indicate that *S. epidermidis* 9 produces a lipase of 97 kDa, as determined by SDS-PAGE, which is sequentially degraded by *S. epidermidis* proteolytic activity to a 43 kDa form. It is likely that undetectable levels of proteolytic activity in medium B caused limited lipase degradation.

A similar result was obtained with *Staphylococcus hyicus* lipase (Gotz *et al.*, 1985; Van Oort *et al.*, 1989). The *S. hyicus* lipase gene was cloned and expressed in *Staphylococcus carnosus*. Although a 71 kDa polypeptide was predicted from the nucleotide sequence, the molecular mass of the lipase as determined by SDS-PAGE was 86 kDa. Active lipases in the range 86–46 kDa were observed during purification procedures. In *S. hyicus* cultures, a 46 kDa lipase was detected. It was concluded that *S. hyicus* lipase is secreted as a pro-protein of 86 kDa and that a hydrophilic N-terminal region of 230 residues is required for efficient secretion of the lipase. Proteolytic cleavage of the pro-lipase occurs with the formation of a 46 kDa mature lipase in *S. hyicus* cultures.

Semi-quantitative methods were developed during this study for the detection of lipase-expressing recombinants. Agars containing lipase substrates were used for overlaying plaques or for plating *E. coli* directly. A combination of tributyrin and triolein agars was used. Although the former is very sensitive to lipase activity, it is not specific, as esterases cause its hydrolysis. Triolein was therefore used for confirming lipase activity. This approach may be used for cloning lipase genes from other sources.

Information obtained from this investigation will facilitate the construction of lipase-deficient isogenic mutants of *S. epidermidis* 9 by allele-replacement mutagenesis. Studies comparing lipase-negative mutants with an otherwise isogenic strain will provide information concerning the ecological and possible pathogenic role of the lipase.

We are grateful to E. Ingham and G. Dougan for antiserum production and assistance with minicell experiments, respectively. The amino acid sequencing was performed by the SERC amino acid sequencing service at the University of Leeds. This work was financially supported by the Leeds Foundation for Dermatological Research.

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