Propionibacterium acnes*, a resident of lipid-rich human skin, produces a 33 kDa extracellular lipase encoded by *gehA

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Five independent clones of the *Propionibacterium acnes* P-37 lipase gene (*gehA*) were obtained in *Escherichia coli*, and the gene was localized to a 2.75 kb *Xho*I fragment by subcloning. The five clones were shown to contain the same gene by Southern blotting with a DIG-labelled probe to *gehA*. The nucleotide sequence of *gehA* was determined, and shown to contain a single ORF of 1017 kb, encoding a protein of 339 amino acids. The predicted molecular mass was 36 kDa. A 33 kDa (PAGE) radiolabelled polypeptide was detected from *E. coli* minicell preparations harbouring *gehA*, which could correspond to GehA after cleavage of the putative 26 amino acid residue signal peptide. *gehA* was overexpressed in *E. coli* under the control of the bacteriophage T7 promoter, and the corresponding polypeptide was found to be present in insoluble aggregates. Active lipase was produced when the overexpressing strain was incubated at a reduced temperature in the presence of sucrose. Purification of lipase from *P. acnes* culture supernatant fluids confirmed the production of a 33 kDa (PAGE) lipase.

Keywords: acne, colonization factor, lipase, molecular cloning, nucleotide sequence

INTRODUCTION

Propionibacterium acnes is a major inhabitant of adult human skin and high population densities are associated with skin sites possessing high numbers of sebum-excreting sebaceous follicles (McGinley *et al.*, 1978). The population densities of these bacteria are low in children, who have low sebum-excretion rates (Leyden *et al.*, 1975), and in adults who have received *cis*-retinoic acid treatment for the follicular disease acne (King *et al.*, 1982). *cis*-Retinoic acid has no direct antimicrobial activity, but does drastically reduce sebum-excretion rate. These findings strongly implicate skin lipids, of which triglycerides are a significant component (Nicolaides & Wells, 1957), in the colonization of human skin by *P. acnes*. It follows that the lipase (EC 3.1.1.3) of *P. acnes* may have an important role in colonization. Furthermore, in the 1960s and 1970s the lipase was implicated as a virulence factor in acne, though more recent investigations disproved this hypothesis (Holland *et al.*, 1981; Eady & Ingham, 1994). Even so, the

organism is considered to be one of the factors involved in the development of acne (Holland, 1989). The bacterium has also been implicated in other diseases, all thought to be initiated from the organisms' colonization of skin (Eady & Ingham, 1994).

P. acnes produces a number of extracellular enzymes including lipase, which has been partially purified from strain P-37 (Ingham *et al.*, 1981). Recently, Gribbon *et al.* (1993) reported that *P. acnes* cell-cell adhesion, and adhesion to the internal surfaces of a continuous culture vessel, occurred in the presence of the free fatty acid (FFA) oleic acid. Gribbon *et al.* (1993) proposed that triglycerides within nascent sebum, which contains no FFA, were partially converted to FFA by lipase, assisting bacterial adherence and colonization of the sebaceous follicle.

This investigation was initiated to determine the role of *P. acnes* lipase in terms of nutrition and/or colonization. This may be studied by comparing a mutant which is deficient in lipase activity with wild-type *P. acnes*, using *in vivo* skin models such as that described by Kearney *et al.* (1982a, b). The use of allele replacement in order to replace a wild-type gene with an interrupted form of the

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same gene has been used to create a number of otherwise isogenic bacterial mutants (Mekalanos *et al.*, 1983; Pizza *et al.*, 1989). The initial stages of allele replacement, namely the cloning and expression of the *P. acnes* lipase gene, are reported in this communication. The nucleotide sequence of *gehA* and the re-purification of lipase from *P. acnes* are also reported.

METHODS

Bacterial strains, plasmids and culture conditions. A laboratory strain of *Propionibacterium acnes* (Type I of Marples & McGinley, 1974) designated P-37 was used (Gribbon *et al.*, 1993). All media were obtained from Oxoid. *P. acnes* was grown in Brain Heart Infusion medium containing 0.5% (w/v) glucose at 34 °C, and was incubated anaerobically under an atmosphere of N₂/CO₂/H₂ (80:10:10, by vol.) in a Microflow anaerobic cabinet (MDH). The strains of *E. coli* and plasmids used are shown in Table 1. *E. coli* was routinely grown under aerobic conditions at 37 °C in LB medium or on LB agar. *E. coli* DS410 was propagated in Brain Heart Infusion medium. When appropriate, ampicillin (100 µg ml⁻¹), tetracycline (12.5 µg ml⁻¹) or chloramphenicol (34 µg ml⁻¹) was added to the medium.

Lipase assay. Lipase activity was assayed by measuring the release of oleic acid from triolein using the method of Ingham *et al.* (1981), except the base for titration (tetra-*n*-butylammonium hydroxide) was used at a concentration of 1.25%

(w/v) in methanol. Lipase activity units were measured as µmol oleic acid produced min⁻¹.

***P. acnes* genomic DNA preparation (Tipper *et al.*, 1993).** *P. acnes* P-37 was grown in tryptone (20 g l⁻¹), yeast extract (10 g l⁻¹), glucose (5 g l⁻¹) medium for 48 h. Cultures were supplemented during late exponential growth (OD₆₀₀ 2.8) with a subminimal bactericidal concentration of penicillin G (20 µg ml⁻¹). Cultures were incubated for a further 4 h and harvested by centrifugation (6800 g, 10 min at 4 °C). Harvested cells were resuspended in 0.025 vol. TE buffer (10 mM Tris, 1 mM EDTA) and SDS was added to 1% (w/v). The cells were incubated at 37 °C for 60 min, predigested pronase (Boehringer) was added to give a final concentration of 0.1% (w/v), and incubation was continued for a further 16 h. Genomic DNA was extracted with phenol/chloroform, ethanol precipitated, and dissolved in TE buffer.

Plasmid DNA preparation and transformation. Plasmid DNA was prepared from *E. coli* using the Magic Minipreps DNA purification system (Promega) or by CsCl density gradient centrifugation. Transformation of *E. coli* was performed by the method of Hanahan (1985).

DNA manipulations. Basic techniques were performed as described by Sambrook *et al.* (1989). Restriction enzymes were obtained from Boehringer Mannheim, Gibco BRL, Pharmacia or Northumbria Biologicals (NBL). T4 DNA ligase was obtained from Gibco BRL and calf intestinal alkaline phosphatase was obtained from Boehringer Mannheim. Restriction-enzyme-generated fragments were separated on 0.8%

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Relevant genotype/phenotype	Source/reference
<i>Escherichia coli</i>		
BL21(DE3)	<i>hsdS gal (λcIts857 ind1 Sam7 nin5 lacUV5-T7 gene 1)</i>	Studier & Moffat (1986)
C600	<i>lac thr leu thi tonA hsdR hsdM</i>	Appleyard (1954)
DH5α	<i>supE44 ΔlacU169 (φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Hanahan (1983)
DS410	<i>minA minB rpsL</i> ; minicell producer	Dougan & Sherratt (1977)
HMS174	<i>recA1 hsdR rif^r</i>	Studier & Moffat (1986)
HMS174(DE3)pLysS	<i>recA1 hsdR rif^r (λcIts857 ind1 Sam7 nin5 lacUV5-T7 gene 1) pLysS (T7 gene 3.5 φ3.8 promoter Cm^r)</i>	Studier (1991)
TG1	<i>supE hsdΔ5 thi Δ(lac-proAB) F' (traD36 proAB⁺ lacI^a lacZΔM15)</i>	Gibson (1984)
XL-1 Blue	<i>supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac-F' [proAB⁺lacI^a lacZΔM15 Tn10(tet^r)]</i>	Bullock <i>et al.</i> (1987)
Plasmid		
pBR322	Ap ^r Tc ^r ; cloning vector	Bolivar <i>et al.</i> (1977)
pBluescript (SK +)	Ap ^r ; cloning vector	Stratagene
pIC20H	Ap ^r ; cloning vector	Marsh <i>et al.</i> (1984)
pIC20R	Ap ^r ; cloning vector	Marsh <i>et al.</i> (1984)
pT7-7	Ap ^r ; T7 expression vector	Tabor & Richardson (1985)
pUL6000	Ap ^r Geh ⁺ ; 6.0 kb <i>Sau3AI</i> <i>P. acnes</i> DNA fragment cloned in pBluescript	This study
pUL6001/pUL6002	Ap ^r Geh ⁺ ; 2.75 kb <i>XhoI</i> fragment from pUL6000 cloned in pBluescript in both orientations	This study
pUL6003/pUL6004	Tc ^r Geh ⁺ ; 1.25 kb <i>BstEII-ScaI</i> fragment cloned into <i>ScaI</i> site of pBR322 in both orientations	This study
pUL6005/pUL6006	Tc ^r Geh ⁻ ; 1.0 kb <i>EcoRV-ScaI</i> fragment cloned into <i>ScaI</i> site of pBR322 in both orientations	This study
pUL6010	Ap ^r Geh ⁺ ; <i>gehA</i> cloned under control of the T7 promoter in pT7-7	This study

agarose gels, then isolated and purified by using the GeneClean II kit (Bio 101). DNA hybridization experiments were performed using the DIG DNA Labelling and Detection kit (Boehringer Mannheim). The 0.53 kb *EcoRV*–*HindIII* fragment isolated from pUL6000 was labelled with digoxigenin-dUTP and used as a probe. Oligonucleotide primers were prepared using an Applied Biosystems oligonucleotide synthesizer and deprotected according to the manufacturer's instructions. PCR was carried out using a *GeneAmp* PCR Core Reagents Kit (Perkin Elmer Cetus) with *AmpliTaq* DNA polymerase (Perkin Elmer Cetus).

Cloning of the lipase (glycerol-ester hydrolase A; *gehA*) gene. Genomic DNA from *P. acnes* was partially digested with *Sau3AI*, and the fragments (5–12 kb) were collected from an agarose gel (0.8%) by electroelution. The *P. acnes* DNA fragments were ligated into *Bam*HI-digested pBluescript SK(+), previously treated with calf intestinal alkaline phosphatase for 30 min at 37 °C. The ligation mixture was used to transform competent *E. coli* DH5 α cells, and these were plated on tributyrin agar supplemented with ampicillin (Farrell *et al.*, 1993). Plates were incubated for 16 h at 37 °C and held at 4 °C. Putative lipase activity was indicated by a zone of clearing in the tributyrin emulsion around colonies after 54 h at 4 °C.

Confirmation of lipase activity of clones. Lipase activity was confirmed using modified triolein medium (Farrell *et al.*, 1993) comprising Blood Agar Base (4%, w/v), Victoria blue B (50 μ g ml⁻¹) and triolein (0.3%, w/v). Triolein was prepared as an emulsion (triolein, 20%, w/v; gum acacia, 2%, w/v; emulsified in distilled water), and added to the molten medium prior to autoclaving. Victoria blue B was dissolved in ethanol (20 mg ml⁻¹), and added to the medium prior to pouring. Lipase activity was indicated by a blue zone of precipitation around colonies.

Nucleotide sequencing and analysis. For nucleotide sequencing, the 2.75 kb *Xho*I fragment from pUL6000 was ligated into *Xho*I-digested pBluescript SK(+) in both orientations. Progressive unidirectional deletions of the inserted DNA were created using the Exonuclease III/mung bean nuclease deletions kit (Stratagene). Sequencing was performed by the dideoxy chain-termination method (Sanger *et al.*, 1977) using the T⁷Sequencing kit (Pharmacia). When appropriate, Deaza G/A T⁷Sequencing Mixes (Pharmacia) were used to aid the resolution of ambiguous sequence data. The Owl amino acid sequence composite database (release 24.0) was searched for amino acid sequence similarities with *GehA* using SWEEP version 4.00.

SDS-PAGE and Western immunoblotting. SDS-PAGE was carried out as described by Laemmli (1970). Western immunoblotting was carried out with the Pharmacia Novablot apparatus as described in the manual. Rabbit anti-*P. acnes* P-37 lipase antiserum was prepared by Ingham *et al.* (1984), and absorbed with a concentrated extract of *E. coli* DH5 α before use. Horseradish-peroxidase-conjugated swine anti-rabbit globulins were from Dako.

Expression of *P. acnes* lipase in *E. coli* minicells. Intact *gehA* (1.25 kb *Bst*EII–*Sca*I fragment from pUL6000, blunt-ended with mung bean nuclease) and a form of *gehA* with the 5'-flanking region and first 96 nucleotides removed (1.0 kb *EcoRV*–*Sca*I fragment) were cloned into the *Sca*I site of pBR322. The fragments were inserted in both possible orientations within the β -lactamase gene, resulting in the disruption of ampicillin resistance. Tetracycline resistance was used for selection. The plasmids were designated pUL6003, pUL6004 (*gehA* positive) and pUL6005, pUL6006

(*gehA* negative), and were used to transform *E. coli* minicells strain DS410. Minicells were prepared and labelled with [³⁵S]methionine as described by Dougan & Sherratt (1977). Plasmid-encoded polypeptides were analysed by SDS-PAGE, followed by autoradiography or Western immunoblotting.

Overexpression of *P. acnes* lipase. PCR was used to incorporate a *Nde*I site at the translational start codon (ATG) of *gehA*, and a *Sac*I site downstream of the translational stop codon. The primers for PCR amplification were 5'-GCGCG-CATATGAAGATCAACGCACGATTTCG-3' and 5'-CGGC-GAGCTCGGCGACTGGCTGCA-3'. The amplification was for 30 cycles using an annealing temperature of 55 °C, 3 mM MgCl₂, 20% (v/v) glycerol and 10 ng template (pUL6001). The *gehA* ORF was placed under the control of the bacteriophage T7 gene 10 promoter by ligation of the 159 bp *Nde*I–*Eco*RI PCR-amplified fragment and the 932 bp *Eco*RI–*Sca*I cloned DNA fragment from pUL6000 into *Nde*I and *Sma*I-digested pT7-7, previously treated with calf intestinal alkaline phosphatase. The plasmid was designated pUL6010. The *E. coli* strain HMS174(DE3)pLysS was transformed with pT7-7 or pUL6010, and plated on tributyrin agar. Colonies were used to inoculate LB medium supplemented with 0.0 or 0.45 M sucrose and were cultured at 37 °C or 27 °C. Cultures were induced in exponential phase growth (OD₆₀₀ 0.6–1.0) with 0.1 or 0.4 mM IPTG. The cells were harvested by centrifugation (7800 g, 10 min at 4 °C), resuspended in 0.03 vol. MES buffer (pH 6.0), and lysed by ultrasonic disintegration. Volumes (10 μ l) were applied to 4 mm wells cut into a tributyrin-agarose plate, comprising tributyrin emulsion (2%, w/v; Farrell *et al.*, 1993), citrate/phosphate buffer (pH 6.5; 0.1 M) and agarose (0.6%, w/v). Plates were incubated for 20 h at 37 °C. Cell extracts were analysed by SDS-PAGE and Western immunoblotting.

Production and purification of *P. acnes* lipase. *P. acnes* P-37 was grown anaerobically in Brain Heart Infusion broth, supplemented with glucose (final concentration 0.5%, w/v) at 34 °C for 65 h. Cells were removed from cultures by filtration (0.3 μ m pore size), and the supernatant fluid was concentrated and desalted by ultrafiltration (10 kDa cut-off membrane). Cation exchange was performed using CM-Sepharose. Proteins were eluted in 50 mM sodium phosphate buffer (pH 6.0), with a linear NaCl gradient of 0.0–0.5 M. Affinity chromatography was performed using heparin-Sepharose and proteins were eluted as before.

RESULTS

Lipase activity of *P. acnes* P-37

In order to confirm the production of extracellular lipase by *P. acnes* P-37, crude culture supernatant fluid was assayed for lipase activity. Extracellular lipase was produced from cells in the post-exponential phase of growth and the lipase concentration remained constant (0.17 U ml⁻¹) after 60 h incubation.

Cloning of the *P. acnes* lipase gene in *E. coli* DH5 α

In order to isolate the lipase gene from *P. acnes* P-37, a pBluescript-based plasmid library containing *P. acnes* genomic DNA inserts was constructed and screened for lipase activity in *E. coli* DH5 α . An estimated total of 10000 recombinant *E. coli* colonies harboured plasmids which contained *P. acnes* genomic DNA. Five putative

Fig. 1. Restriction endonuclease cleavage site map of pUL6000. Nucleotides are numbered from the junction of the *Bam*HI site of pBluescript and the terminal *Sau*3AI sites in the *P. acnes* DNA. The exact locations of restriction sites within pBluescript SK(+) are marked. The locations within *P. acnes* DNA are approximate. The locations of the ampicillin gene (ApR) and the origin of replication (Ori) are marked. The minimum region of DNA required for lipase activity deduced by subcloning is indicated in black. Restriction sites marked with an asterisk were deduced from sequence data and other sites may exist within pUL6000. Unfilled area, pBluescript; hatched area, cloned DNA; B/S, *Bam*HI/*Sau*3AI.

lipase clones were detected by observation of a zone of clearing in the tributyrin emulsion surrounding *E. coli* colonies. Putative lipase activity was detected after overnight incubation at 37 °C followed by 54 h at 4 °C. Although very sensitive to lipase activity, tributyrin is not a specific lipase substrate as it is also hydrolysed by esterases. Therefore, it was necessary to confirm lipase activity using triolein, a less sensitive but specific lipase substrate. *E. coli* strains XL1-Blue, TG1, DH5 α and C600 were transformed with plasmid DNA isolated from the five putative lipase clones. The expression of putative lipase activity within the four strains of *E. coli* was estimated by measuring the size of the zone of clearing in the tributyrin emulsion surrounding individual colonies. *E. coli* strain TG1 produced the largest zone of clearing in the tributyrin emulsion with respect to colony size (data not shown), and was freshly transformed with pBluescript DNA and plasmid DNA isolated from each of the five putative lipase clones. The resultant transformants were plated on triolein medium. Lipase activity was detected from all the five putative lipase clones, but not from the pBluescript control.

The quantitative assay was not sufficiently sensitive to detect lipase activity within concentrated cell extracts. The limit of sensitivity of the assay was 0.003 U ml⁻¹ (data not shown). Furthermore, SDS-PAGE revealed no

		RBS	
TTTTTGTAGGCTACCCCTTTTCGAATTGAATCAATGATTACGAAATTCGCCCGTAGTTCCGCTAGGAGTCA	75		
M K I N A R F A V M A A S V A V L M A A P I A	24		
CTGATGAAGATCAACGACGATTCGCGTCATGCGCGGAGTGTGGCGTCTGTATGGCTGCGCGCGGATTCGCG	150		
Q A A T S P G D I H P L V Q A A H S P D G I P G N	49		
CAGGCTGCTACTTCGCGGGGATATCCATCCCTGTGTCAGGACGACGACGCGGATTCGCGGTAAC	225		
\EcoRV			
G V G P E F H T S S M A R S Y S E K H L G V A P R	74		
GGCGTGGCGGGAATTCATACGTGTCGATGGCGGTCTCTACAGCAGAGACCTGGCGGTGGCGCGCGG	300		
\EcoRI			
G V N D F S C K V K P G D R P V I L I P G T G G N	99		
GGTGTGAACGACTTCTCTCAAGGTCAAGCCCGGCGACCGCGGTATCTGATTCGCGGTATGGCGGCAAT	375		
A F A T W S F Y G P H L A H E G Y C V Y T F T T N	124		
GCGTTCGATCGGTCGCGAAGCGACGGCTCTGAGAGGTCAGGCGGTATGTCACACCTTCACCGCAAT	450		
V P V G I L D E G W G F T G D V R A S A Q A L G A	149		
GTCCCGTGGGGATCTCGACGAGGCTGGGGCTTCACCGGTGACGTGCGCGCTCCGCGAGGCGTGGGTGCC	525		
F V D R V R K A T G S E K V D F V G H S Q G G I	174		
TTGTGGATCGGTCGCGAAGCGACGGCTCTGAGAGGTCGACTTCGCGACATTCGACGGTGGCGGATC	600		
L P N A Y I K M Y G G A S K V D K L I G L V A A N	199		
CTGCCGAACGCTACATCAAGATGTATGGCGGAGCGTCAAGGTGACAGCTGATCGGATTCGCGCGCAAC	675		
H G T T A V G L D K L V D G L P E A V K D F L S T	224		
CACGGCACCGCGCTCGTCTCGACAAGCTTGTGACGGCTGCGCGAGGCTGTGAGGATTTCTTAGCACG	750		
\HindIII			
W S Y D H N M E A Y G Q Q L K G S A L M Q Q V Y R	249		
TGGAGTACGACACACATGAGGCGCTACGGCGACGCTCAAGGATCAGCGTTGATGCAACAGGTTTACGCT	825		
D G D T V P G I A Y T V I S T R L D M T V T P Y T	274		
GACGGGACACGCTCCCGGTATCGCCTACCGCTCATCTCCACCGCTCGACATGACGCTACGCGCTACAG	900		
Q A F L K G A K N M T V Q D A C P L D A Y G H G R	299		
CAAGCTTCTCAAGGGCGCCAGAACATGACCGTGCAGGAGCGCTCCCTCTGGATGCTATGCCACCGCGCG	975		
L P Y D P V A Y Q M V L N A L D P N H P R E I S C	324		
CTACCTATGATCCCGTCGCTACAGATGGTGCTCAACGCCCTCGATCGCAACACCCACGAGAGATCTCTGC	1050		
T W R P R V L P V S T T D A A *	339		
ACGTGGCGGCGCGGTTCTGCGGTATCCACCGGATGCTGATGACACTGTGGCAGGTGAGGGTTCTCCAGCG	1125		
CGTGGCGGCTGGCCACCAACACGACTGTGGTGACAACGACGACTCGTCCCTGCGCTTGTGCGCGGTG	1197		
\ScaI			

Fig. 2. Complete nucleotide sequence of the *P. acnes* lipase gene, *gehA*, and the deduced amino acid sequence of the lipase. The nucleotide data shown start from nucleotide 601 of the 2.75 kb *Xho*I fragment. The putative ribosome-binding site (RBS) is underlined. The 'lipase consensus sequence' (G-H-S-Q-G) and the putative signal peptidase recognition site (A-Q-A) are in bold. Restriction sites for *Eco*RV, *Eco*RI, *Hind*III and *Sca*I are indicated. A sequence capable of forming a hairpin-loop structure which may act as a terminator of transcription is doubly underlined.

additional polypeptide species within cell extracts of the lipase clones when compared to an extract of cells harbouring pBluescript (data not shown).

Physical mapping and subcloning of the cloned gene

Plasmid DNA was isolated from the five lipase clones (A-E) and the smallest (A, 9.0 kb) was designated pUL6000. The restriction map of pUL6000 is shown in Fig. 1. To localize the lipase gene (*gehA*) in pUL6000, a series of subclones was constructed in the plasmid vectors pBluescript or pIC20R. The resultant plasmids were transformed into *E. coli* and tested for the expression of lipase, using tributyrin medium. A 2.5 kb *Xho*I-KpnI DNA fragment was common to all the fragments which encoded lipase activity (Fig. 1). The 2.75 kb *Xho*I fragment was subcloned in both possible orientations into the *Xho*I site of pBluescript, forming pUL6001 and pUL6002. The lipase activity expressed in *E. coli* from pUL6001 or pUL6002 was estimated on tributyrin medium, and shown to be independent of the orientation of the lipase gene (*gehA*) with respect to the

Table 2. Comparison of the amino acid sequence of GehA with other bacterial lipases, surrounding the 'lipase consensus sequence'

The catalytic serine residue is in bold. Amino acid residues conserved in at least six of the ten sequences are highlighted.

Organism*	Amino acid residue													
<i>Propionibacterium acnes</i>	G	S	E	K	V	D	F	V	G	H	S	Q	G	G
<i>Staphylococcus epidermidis</i> ¹	P	G	Q	K	I	H	L	V	G	H	S	M	G	G
<i>Staphylococcus aureus</i> ²	P	G	K	K	V	H	L	V	G	H	S	M	G	G
<i>Streptomyces lividans</i> ³	D	A	T	R	L	G	V	M	G	H	S	M	G	G
<i>Bacillus subtilis</i> ⁴	G	A	K	K	V	D	I	V	A	H	S	M	G	G
<i>Pseudomonas glumae</i> ⁵	G	A	T	K	V	N	L	I	G	H	S	Q	G	G
<i>Pseudomonas cepacia</i> ⁶	G	A	T	K	V	N	L	V	G	H	S	Q	G	G
<i>Pseudomonas aeruginosa</i> ⁷	G	Q	P	K	V	N	L	I	G	H	S	H	G	G
<i>Pseudomonas fluorescens</i> ⁸	H	G	K	D	V	V	V	S	G	H	S	L	G	G
<i>Moraxella</i> TA144 (lip2) ⁹	S	P	S	R	I	V	L	S	G	D	S	A	G	G

*References: 1, Farrell *et al.* (1993); 2, Lee & Iandolo (1986); 3, Pérez *et al.* (1993); 4, Dartois *et al.* (1992); 5, Frenken *et al.* (1992); 6, Jørgensen *et al.* (1991); 7, Wohlfarth *et al.* (1992); 8, Tan & Miller (1992); 9, Feller *et al.* (1991).

plasmid (*lac*) promoter (data not shown). These results indicated that transcription of *gehA* was from the *P. acnes* promoter.

Nucleotide sequencing of *gehA*, and its predicted amino acid sequence

The nucleotide sequence of the 2.75 kb *Xho*I DNA fragment was determined from both strands by the dideoxy chain-termination method. The complete nucleotide sequence of the *P. acnes gehA* gene including the 5' and 3' flanking regions and the deduced amino acid sequence of the lipase is shown in Fig. 2. A single ORF was detected which extended from nucleotides 679 to 1695 of the 2.75 kb *Xho*I DNA fragment, and encoded a polypeptide of 339 amino acid residues with a predicted molecular mass of 35993 Da. The predicted translation initiation codon starting at nucleotide 679 is preceded by a potential ribosome-binding site (GAGGAG). The first 26 amino acid residues in the translation product of *gehA* (GehA) were characteristic of a signal peptide. This region contained three very hydrophilic residues in the first six, followed by 20 residues, of which 17 were hydrophobic. The postulated signal peptide ended in the sequence Ala-Gln-Ala, an expected sequence immediately preceding the cleavage site for a signal peptidase (Perlman & Halvorson, 1983). The predicted molecular

mass of the signal peptide was 2617 Da. Cleavage of the postulated signal peptide during translocation across the cytoplasmic membrane would result in a predicted molecular mass of 33376 Da for the mature form of *P. acnes* extracellular lipase. The G+C content of the *gehA* gene was 63.7%. The codon usage of *gehA* was non-random and, as expected for an organism with a high G+C content, G or C was highly favoured (80%) in the third position. The region upstream of the translation initiation codon had little sequence similarity with previously characterized promoter sequences. The region of DNA downstream of the translational stop codon contained a sequence capable of forming a hairpin-loop structure ($\Delta G = -9.2 \text{ kcal mol}^{-1}$; $-38.6 \text{ kJ mol}^{-1}$), which could act as a weak terminator of transcription.

Comparison of the amino acid sequence of GehA with other lipases

Comparison of the predicted amino acid sequence of GehA with proteins within the Owl composite database indicated a low degree of similarity with previously reported non-lipase sequences. The greatest similarity was observed between *P. acnes* lipase and other lipases. The highest score obtained with the whole GehA sequence was with the lipase from *Bacillus subtilis*,

Fig. 3. Analysis of the five lipase clones by Southern blotting using a DIG-labelled probe to *gehA*. (a) Plasmid or *P. acnes* genomic DNA was digested with *KpnI* and subjected to agarose gel electrophoresis (1%, w/v). (b) DNA was transferred to a nylon membrane and hybridized with a probe to *gehA*. Lanes (lanes 2–8 are digested with *KpnI*): 1, λ HindIII standards; 2, pUL6000; 3, clone B; 4, clone C; 5, clone D; 6, clone E; 7, pUL6000 (1/10 dilution); 8, *P. acnes* DNA. The 2.8 kb *KpnI* fragment which hybridized with the *gehA* probe is marked with an arrow.

which had 37.8% identity over a 74-residue overlap (Dartois *et al.*, 1992). The highest amino acid sequence similarities were observed in a region containing a pentapeptide 'lipase consensus sequence', G-X₁-S-X₂-G. In addition to the conserved residues within this pentapeptide, the immediately preceding residues within GehA agree with the lipase motif defined within the PROSITE dictionary of protein sites and patterns (Bairoch, 1992), which comprises the residues (L,I,V)-X-(L,I,V,F,Y)-(L,I,V,T)-G-(H-Y)-S-X-G. Table 2 shows an alignment of the predicted amino acid sequence of *P. acnes* lipase (GehA) and those of other bacterial lipases in this region.

Comparison of the five lipase clones by hybridization

In order to determine whether all five of the lipase clones obtained from the *P. acnes* genomic library contained the same lipase gene, *gehA*, a DIG-labelled probe was prepared from the 530 bp *EcoRV*-*HindIII* DNA fragment located within the *gehA* ORF. Fig. 3(a) shows an agarose gel of *KpnI*-digested plasmid DNA isolated from the five lipase-positive clones (pUL6000, and plasmids isolated from clones B–E) and similarly digested *P. acnes* genomic DNA. Fig. 3(b) shows the results of Southern transfer of DNA from the gel to a nylon membrane, followed by hybridization with the *gehA* probe at 68 °C for 16 h. The *gehA* probe hybridized with a single 2.8 kb *KpnI* fragment in all of the lipase clones and the *P. acnes* genome. These results indicated that a single copy of *gehA* was present in the *P. acnes* genome.

Expression of GehA in *E. coli* minicells

In order to determine the molecular mass of GehA when expressed in *E. coli*, a minicell-producing strain of *E. coli* (DS410) was used for the detection of *gehA*-encoded polypeptide(s). Plasmids containing intact or truncated *gehA* were constructed in pBR322. The *ScaI* site of pBR322 was used as insertion of DNA at this site would disrupt the β -lactamase gene. Minicells harbouring pBR322, pUL6003, pUL6004, pUL6005 or pUL6006 were purified, and plasmid-encoded polypeptides were labelled with [³⁵S]methionine. Fig. 4 shows an autoradiograph of radiolabelled minicell preparations subjected to SDS-PAGE. Inactivation of the β -lactamase gene in pUL6003–pUL6006 prevented the expression of the three previously characterized β -lactamase polypeptides (25, 28, 30 kDa; Covarrubias *et al.*, 1981) which could be detected in the pBR322 preparation. The 34 kDa product of the tetracycline resistance gene and the 10 kDa polypeptide of unknown function were detected in all the five extracts, as previously reported by Covarrubias *et al.* (1981). A 33 kDa polypeptide was detected in the extracts of minicells harbouring either pUL6003 or pUL6004, but was not detected in the three control minicell extracts. The expression of this polypeptide was shown to occur when the fragment was cloned in either orientation. Approximately 160 bp *P. acnes* DNA was cloned upstream of the putative ATG translational start codon, indicating that the gene was transcribed from a promoter located within this region. This 33 kDa polypeptide was presumed to be the product of *gehA*, and the molecular mass possibly indicated cleavage of the signal peptide. However, in order to show that the signal peptide was indeed cleaved

Fig. 4. Plasmid-encoded polypeptides detected by SDS-PAGE (9%, w/v) and autoradiography of purified and [35 S]methionine-labelled *E. coli* DS410 minicells harbouring the indicated plasmids. Lane 1, Coomassie-Brilliant-Blue-stained polypeptide molecular mass markers. Lanes 2–6 correspond to minicells harbouring the plasmid indicated: 2, pBR322; 3, pUL6003 (*gehA* positive); 4, pUL6004 (*gehA* positive); 5, pUL6005 (*gehA* negative); 6, pUL6006 (*gehA* negative). Lipase is indicated by the arrow at 33 kDa.

from *GehA* when expressed in *E. coli*, it would be necessary to obtain further evidence such as N-terminal microsequencing data.

Overexpression of *gehA*

In order to increase the level of expression of *P. acnes* lipase from *E. coli*, the gene was placed under the control of the powerful bacteriophage T7 gene 10 promoter in the construct pUL6010. Cell extracts of *E. coli* HMS174(DE3)pLysS harbouring pT7-7 or pUL6010 were analysed at various times after induction of T7 RNA polymerase. An additional polypeptide (33 kDa) was detected in extracts of *E. coli* harbouring pUL6010, following induction with IPTG. This polypeptide was first detected at 30 min post-induction and then accumulated between 30 and 180 min post-induction (data not shown). Lipase activity was not detected in cell extracts containing significant amounts of this polypeptide. SDS-PAGE of the supernatant and pellet fractions of the extract indicated that the 33 kDa lipase was present in the insoluble fraction in the form of inclusion bodies (data not shown). In order to express lipase from pUL6010 in *E. coli* in a soluble and active form, the incubation temperature and growth medium were varied. The lipase activity of cell extracts harbouring

Fig. 5. Photograph of wells from a tributyrin agarose plate (pH 6.5) loaded with 10 μ l samples of HMS174(DE3)pLysS-(pUL6010) cell extracts from cultures grown under the indicated conditions. The plate was incubated for 20 h at 37 °C, and lipase activity was observed as zones of clearing in the tributyrin emulsion.

pUL6010 were measured semi-quantitatively on a tributyrin-agarose plate (Fig. 5). Lipase activity was greatest in extracts from cultures grown at 27 °C in the presence of 0.45 M sucrose. A small amount of active lipase was also expressed from cultures which were grown at 37 °C in LB with 0.45 M sucrose. In order to demonstrate that lipase expression was from the *P. acnes* gene, cultures of *E. coli* harbouring pUL6010 or pT7-7 were grown in the presence of 0.45 M sucrose at 27 °C. The corresponding cell extracts contained 3.0 and 0.0 U lipase activity ml^{-1} , respectively.

Polypeptides expressed in *E. coli* from pUL6010 were compared to a pT7-7 control by Western blot analysis with antiserum raised to partially purified *P. acnes* lipase (Fig. 6). A 33 kDa polypeptide within both the supernatant and pellet fractions of the extract of *E. coli* harbouring pUL6010 reacted with the antiserum. This polypeptide was absent from the extract of cells harbouring the control plasmid, pT7-7. An additional cross-reactive polypeptide (61 kDa) was detected in the supernatant fractions of the extracts harbouring either pUL6010 or the pT7-7 vector control.

Production and purification of *P. acnes* P-37 lipase

P. acnes lipase was produced as a 33 kDa polypeptide in the heterologous host *E. coli*. In order to determine the molecular mass of the enzyme secreted by *P. acnes*, the enzyme was purified to near homogeneity from crude culture supernatant fluid.

Fig. 6. Western blot analysis of the expression of recombinant lipase using antiserum raised to partially purified *P. acnes* lipase (Ingham *et al.*, 1984). The antiserum was shown to react with the recombinant *P. acnes* lipase cloned in this study. *E. coli* HMS174(DE3)pLysS extracts harbouring either pT7-7 or pUL6010 were prepared from cultures grown at 27 °C, in LB medium supplemented with 0.45 M sucrose, and induced with 0.1 mM IPTG. Lanes: 1, Indian-ink-stained molecular mass markers (45 kDa standard was not stained by Indian ink, but its position is marked); 2, *P. acnes* supernatant fluid; 3, pUL6010 soluble fraction; 4, pUL6010 insoluble fraction; 5, pT7-7 soluble fraction; 6, pT7-7 insoluble fraction. A substantial proportion of lipase polypeptide is detected within the soluble fraction of the pUL6010 extract.

Crude culture supernatant fluid was collected from late exponential growth phase *P. acnes* cultures (65 h), and was subjected to cation-exchange chromatography. Lipase eluted from the CM-Sepharose as a single peak of activity with 0.32 M NaCl. Analysis of lipolytic fractions by SDS-PAGE indicated the presence of a major polypeptide species of 33 kDa, which was contaminated with other polypeptide species (Fig. 7). To determine whether the 33 kDa polypeptide had lipase activity, an alternative purification schedule was followed. *P. acnes* P-37 culture supernatant was subjected to heparin-Sepharose chromatography, from which lipase eluted as two peaks of activity. SDS-PAGE of the lipolytic fractions from the column demonstrated that the major polypeptide detected within the first peak of activity was 33 kDa and there were very low amounts of other polypeptides detected in fraction 36 (Fig. 7). This polypeptide was also detected in the second peak of activity (Fig. 7), and was present in good proportion to the amount of lipase activity within the fraction. These results indicated that *P. acnes* P-37 produced a single 33 kDa lipase.

DISCUSSION

Cloning and expression of *P. acnes* lipase

In this study, the *P. acnes* lipase gene (*gehA*) was cloned and expressed from its own promoter in *E. coli*, and is only the second protein-encoding gene to be sequenced from a species of the cutaneous propionibacteria. The gene encoding another *P. acnes* extracellular enzyme, hyaluronidase (EC 4.2.2.1), has recently been cloned and sequenced (B. M. Steiner and others; EMBL accession number PA15927). The nucleotide sequence of *gehA* was determined and the G + C content of the gene was shown to be similar to that of the *P. acnes* hyaluronidase gene (64.9% overall, 79.7% in the third position; B. M. Steiner and others, unpublished). The predicted molecular mass of the translation product of *gehA* was 35 993 Da (36 kDa). After cleavage of the postulated signal peptide, the mature protein (GehA) had a predicted molecular mass of 33 376 Da (33 kDa). In addition to the *gehA* gene promoter, promoters from related organisms such as the classical propionibacteria or the corynebacteria have also been shown to function in *E. coli* (Ladror *et al.*, 1991; Marsh *et al.*, 1989; Martin *et al.*, 1987).

In order to increase the production of GehA in *E. coli*, *gehA* was placed under the control of the bacteriophage T7 promoter and expressed using a well-characterized system (Studier & Moffatt, 1986; Studier, 1991). The overexpressed GehA polypeptide was produced as inclusion bodies which were absent from control cell extracts. The irregular shape of these inclusion bodies might support the possibility that GehA was located in the periplasm, as a previous report (Bowden *et al.*, 1991) suggested that periplasmic inclusion bodies could be differentiated from those expressed in the cytoplasm by their irregular morphology. The formation of soluble recombinant proteins in the periplasm of *E. coli* is often favoured by incubation at low temperature (Schein & Noteborn, 1988; Hockney, 1994) or in the presence of non-metabolizable sugars such as sucrose (Bowden & Georgiou, 1988), and a combination of both of these incubation parameters resulted in a marked increase in the production of active lipase polypeptide. Using this system, the 33 kDa recombinant polypeptide was shown to possess lipase activity. Additional evidence such as N-terminal microsequencing data would be required to confirm signal peptide cleavage in *E. coli*.

Does the *P. acnes* genome contain more than one lipase gene?

The partially purified lipase from a previous study contained a major (41.2 kDa) and two minor (67 and 126 kDa) polypeptide species by SDS-PAGE (Ingham *et al.*, 1981). The major (41.2 kDa) polypeptide was assumed to be lipase, and no 33 kDa polypeptide was reported. The discrepancy between the molecular mass of *P. acnes* lipase reported in the previous study (41.2 kDa) and that shown here (33 kDa) could be explained in a number of ways: (i) the 41.2 kDa polypeptide of Ingham *et al.* (1981) was the same as the

Fig. 7. (a) Silver-nitrate-stained SDS-polyacrylamide gel (10%, w/v) of fractions obtained in the purification of lipase from *P. acnes* culture supernatant fluid, and (b) lipase activity (U ml^{-1}) of the respective samples. Lanes: 1, culture supernatant fluid; 2, CM-Sepharose fraction 28 (0.32 M NaCl); 3–12, heparin-Sepharose fractions 33–42. A 33 kDa lipase is nearly pure in fraction 36 from the heparin-Sepharose column. This is the major polypeptide species within CM-Sepharose fraction 28, and the amount of this polypeptide is proportional to the total lipase activity detected within each fraction.

33 kDa lipase identified in this study, and the discrepancy could be explained by the differences in PAGE analyses used in the two studies; (ii) *P. acnes* P-37 produces two lipase polypeptides, the 33 kDa lipase cloned in this study, and a second lipase of 41.2 kDa; (iii) the partially purified lipase preparation contained a number of contaminating polypeptides and the 33 kDa enzyme was present in an amount which was not detected by SDS-PAGE and Coomassie Blue staining.

The first of these possible explanations was eliminated by detection of a 33 kDa polypeptide expressed from *gehA* in *E. coli*, when under the control of either the *P. acnes* gene promoter or the bacteriophage T7 promoter. This result showed that the *GehA* polypeptide behaved as expected when subjected to SDS-PAGE.

In order to determine whether the partially purified lipase preparation (Ingham *et al.*, 1981) contained *GehA*, and/or another lipase polypeptide, antiserum raised in rabbits from this preparation was used. Overproduced recombinant lipase reacted in Western blots with this antiserum, indicating that the 33 kDa cloned lipase was present in the enzyme preparation used to raise the antiserum.

In this study, the purification of lipase from *P. acnes* P-37 supernatant fluid demonstrated that a 33 kDa lipase was produced by this strain. The results did not indicate that a second lipase was produced. Additional polypeptides, including a 43 kDa species which could correspond to the 41.2 kDa polypeptide of Ingham *et al.* (1981), eluted from the heparin-Sepharose column in fractions that formed the second of the two peaks of lipase activity. As fractions from the second peak contained both the 33 and 43 kDa polypeptides, it was not possible to show whether the 43 kDa polypeptide possessed lipase activity. However, there was a strong association of lipase activity with the elution of the 33 kDa polypeptide from the CM-Sepharose and heparin-Sepharose columns, which was not the case for the 43 kDa polypeptide. For example, similar levels of the 43 kDa polypeptide were detected in fractions 40 and 41 (Fig. 7), although lipase activity was threefold lower in fraction 41. In addition, lipase activity was not detected in fraction 42, which contained the 43 kDa polypeptide and no detectable 33 kDa lipase.

The molecular mass discrepancy observed between the two studies is explained by contamination of the

partially purified lipase of Ingham *et al.* (1981) with other polypeptides. The 33 kDa lipase was not detected by Coomassie Blue staining. The results obtained in this study indicate that the *P. acnes* genome contains a single lipase gene, *gehA*, expressed as a single 33 kDa extra-cellular polypeptide, GehA. This is because (i) all five of the lipase clones obtained from the *P. acnes* genomic library contained *gehA*, and (ii) purification of lipase activity from *P. acnes* culture supernatant fluid indicated that only one lipase polypeptide species was produced.

A comparison between *P. acnes* and other lipases

Bacterial lipases have been described as a versatile group of enzymes which share little amino acid sequence homology (Jaeger *et al.*, 1994). The lipase of *P. acnes* (GehA) was shown to be no exception, as significant similarity with other protein sequences was limited to a region which surrounded the postulated catalytic serine residue, the 'lipase consensus sequence'. As a result of the limited similarity between the amino acid sequence of *P. acnes* and other lipases, the Asp(Glu) and His residues which would form the remainder of the catalytic triad were not identified in this study. Further studies are required to determine the two remaining residues of the catalytic triad.

A number of *Pseudomonas* lipases, and possibly the lipase from *Streptomyces* sp. M11, require a second gene product for expression and/or secretion of active lipase (Frenken *et al.*, 1993; Ihara *et al.*, 1992; Pérez *et al.*, 1993). These 'lipase-specific foldases' were shown to be required *in trans* for functional activation of the enzymes, and were encoded by a downstream gene. In the case of *P. acnes* lipase, a similar amount of lipase activity was expressed in *E. coli* irrespective of the presence of substantial (> 2 kb) flanking regions, and no ORF encoding a potential 'foldase' was identified downstream of *gehA* (data not shown). In addition, *P. acnes* lipase did not contain a pro-sequence, an N-terminal amino acid sequence of approximately 30 kDa (including signal peptide) which was proteolytically cleaved in the mature form of staphylococcal lipases (Lee & Iandolo, 1986; Götz *et al.*, 1985; Farrell *et al.*, 1993). The *gehA* nucleotide sequence data and lipase expression studies suggested that GehA was expressed in both *E. coli* and *P. acnes* as a 33 kDa polypeptide.

This investigation will facilitate the construction of a lipase-deficient isogenic mutant of *P. acnes* P-37 by allele replacement mutagenesis when a system for the introduction of foreign DNA into *P. acnes* has been developed. The mutant will be tested for its ability to colonize human sebum-rich skin. Such studies may be carried out using previously described *in vivo* human skin models (Kearney *et al.*, 1982a, b).

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