APPLIED AND ENVIRONMENTAL MICROBIOLOGY, July 1995, p. 2674–2680 Vol. 61, No. 7
0099-2240/95/$04.00+0

Gene Cloning, Sequence Analysis, Purification, and Secretion by *Escherichia coli* of an Extracellular Lipase from *Serratia marcescens*

XUYANG LI,[1] SUSANNE TETLING,[2] ULRICH K. WINKLER,[2] KARL-ERICH JAEGER,[2]* AND MICHAEL J. BENEDIK[1]

Department of Biochemical and Biophysical Sciences, University of Houston, Houston, Texas 77204-5934,[1] *and Lehrstuhl Biologie der Mikroorganismen, Ruhr-Universität, D-44780 Bochum, Germany*[2]

Received 12 January 1995/Accepted 3 May 1995

The gene encoding extracellular lipase of *Serratia marcescens* has been identified from a phage λ genomic library. Formation of orange-red fluorescent plaques on rhodamine B-triolein plates was used to identify phages carrying the lipase gene. A 2.8-kb *Sal*I fragment was subcloned into a plasmid, and lipase was expressed in *Escherichia coli*. Extracellular lipase was detected in the presence of the secretion plasmid pGSD6 carrying the genes *prtD*, *-E*, and *-F*, which guide the secretion of protease from *Erwinia chrysanthemi*. Determination of the nucleotide sequence of the entire cloned fragment revealed an open reading frame coding for a 613-amino-acid protein with a predicted M_r of 64,800. Analysis of the amino acid sequence revealed significant homology (around 70%) to lipases of *Pseudomonas fluorescens* strains. The lipase-specific consensus sequence G-X_1-S-X_2-G resided in the amino-terminal part of the protein, and carboxyl-terminal consensus sequences were an L-X-G-G-B-G-B-B-X repeat motif and a so-called aspartate box, respectively, which are both found in proteins secreted by the class I secretion pathway. Lipase was purified from the supernatant of a culture carrying a lipase expression vector, and analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed an M_r of 64,000 for the purified protein. Our results suggest that the lipase of *S. marcescens* belongs to the group of extracellular enzyme proteins secreted by the class I secretion pathway.

Lipases of gram-negative bacteria have been extensively characterized recently, both biochemically and by molecular genetic studies, because they are considered valuable tools for a variety of biotechnological applications (16). At present, the best-studied group of lipases belong to the genus *Pseudomonas*, with at least 14 different enzymes being described with respect to biochemical properties or DNA sequences of the corresponding genes (16).

Most lipases produced by gram-negative bacteria are secreted via the two-step pathway (16), which for *Pseudomonas aeruginosa* consists of at least 12 different gene products (Xcp proteins) (35). The *P. aeruginosa* lipase contains a 26-amino-acid signal sequence (41) and is secreted via this pathway. However, the lipase of the closely related species *Pseudomonas fluorescens*, which belongs to the same rRNA homology group as *P. aeruginosa* (26), is secreted by the class I secretion pathway (9).

The gram-negative enteric bacterium *Serratia marcescens* also releases an extracellular lipase, as well as a number of other extracellular proteins, into the medium. The class I secretion pathway is in fact also used by *S. marcescens* for the secretion of its extracellular metalloprotease (22, 33). This system consists of three different proteins which form a pore-like structure extending from the cytoplasm through both the inner and outer membranes into the extracellular space (27). In *Escherichia coli*, α-hemolysin is the prototype enzyme using this system which is built up by the two inner membrane proteins, HlyB, belonging to the ABC-binding cassette superfamily of transport proteins, and HlyD and the outer membrane protein TolC (18, 36). All proteins using this system share some characteristic features in that they do not possess an N-terminal signal sequence and they contain up to several repeats of a C-terminal glycine-rich consensus sequence, L-X-G-G-X-G-X-D (6); however, the function of this consensus motif for secretion is still controversial (23). Here we report the cloning of the lipase gene from *S. marcescens* and demonstrate that this lipase also uses the class I secretion pathway.

The physiology and regulation of lipase production by *S. marcescens* have been studied, and results indicate that lipase production was inducible by cyclic AMP and was, at least temporarily, stored at the bacterial outer membrane before it was released into the growth medium (39, 40). Attempts made to purify and biochemically characterize this lipase indicated a strong tendency to form high-M_r aggregates with lipopolysaccharide (LPS) which could be reduced in size by treatment of lipase with detergents such as sodium deoxycholate (24a).

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used are listed in Table 1. Bacteria were grown in solid or liquid LB medium (28). Antibiotics were added at the following concentrations: ampicillin at 100 μg/ml for *E. coli* and 500 μg/ml for *S. marcescens*, kanamycin at 25 μg/ml for *E. coli* and 100 μg/ml for *S. marcescens*, tetracycline at 50 μg/ml, and chloramphenicol at 30 μg/ml. Indicator plates for β-galactosidase were supplemented with 40 μl of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) in *N*,*N*-dimethylformamide (20 mg/ml) and 10 μl of 0.1 M isopropyl-β-D-thiogalactopyranoside (IPTG). Lipase indicator plates were prepared as described elsewhere (20).

Bacteria used for pulse-labelling experiments were grown in MOPS minimal medium (24) made with 0.7% glycerol and 20 μg of each of the 20 amino acids ml^{-1}.

Phage λ-infected cells were plated in 3 ml of top agar (LB with 0.6% agar and 10 mM $MgSO_4$) poured on top of standard LB plates.

Lipase assays. Lipase activity was routinely determined by an assay to measure the amount of *p*-nitrophenol formed from *p*-nitrophenolpalmitate (p-Npp assay) as previously described (32). Lipase activity could also be detected by using rhodamine B-triolein agar plates (20) by directly applying cells to the plates or culture supernatant to preformed holes. Lipase activity was detected under UV light (wavelength, 350 nm) as orange-red fluorescent halos around the colonies or the holes.

* Corresponding author. Phone: (49) 234 700 3101. Fax: (49) 234 709 4114.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Reference or source
Strains		
E. coli		
JM101	*supE thiD* (*lac-proAB*)/F′ *traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15	28
K-12(pHyl152)	Hemolytic wild type	25
S. marcescens SM6	Wild type	10
Plasmids		
pBGS18	Kan^r vector	31
pUC119	Ap^r phagemid vector	28
pGSD6	Secretion functions of *E. chrysanthemi* (*prtD prtE prtF*)	7
pRSC6	Secretion functions of *E. coli* (*hylB hylD*)	30
pSE420	Expression vector with *trc* promoter	5
pBGS18-LipS1	2.1-kb *Sal*I fragment of lipase	This work
pBGS18-Lip05C	Lipase deletion clone Δ05C in pBGS18	This work
pUC118-Lip05C	Lipase deletion clone Δ05C in pUC118	This work
pSE420-Lip05C	Lipase deletion clone Δ05C in pSE420	This work
pBGS18-Lip10F	Lipase deletion clone Δ10F in pBGS18	This work
pSE420-LipRB	Lipase deletion at ribosome binding site in pSE420	This work
pUC118-Lip05C-LacZ	Lipase-LacZ translational fusion	This work
pSE420-Lip05C-LacZ	Lipase-LacZ translational fusion	This work

Plasmid constructions and subcloning. ExoIII deletions were generated for sequencing the lipase gene (28). The deletion clone Lip05C was moved into pBGS18 (31), pUC118, or pSE420 (5) for expression studies by using the unique *Eco*RI and *Hin*dIII sites from the polylinker.

In order to construct pSE420-LipRB, a unique 2.2-kb lipase fragment was generated by PCR amplification by using a primer at the beginning of the lipase coding region which includes an *Nco*I site. The fragment was made blunt with T4 polymerase and ligated into the *Sma*I site of pBGS18. The amino-terminal 400 nucleotides (nt) was then excised by using the polylinker *Eco*RI and internal *Cla*I sites and used to replace the same region in a wild-type lipase clone. In this way, the amino-terminal *Nco*I site was retained but the remainder of the lipase gene was wild type and not from the PCR amplification. The sequence of the 400-nt amplified region was confirmed by DNA sequencing. The *Nco*I-*Sal*I lipase gene was then subcloned into pSE420 to make pSE420-LipRB.

DNA sequencing. Single-stranded DNA sequencing was performed by the dideoxynucleotide chain termination method (29) employing T7 DNA polymerase (Sequenase version 2.0; U.S. Biochemicals) with [^{35}S]dATP and M13 or phagemid single-stranded DNA as the template. Inosine reactions were used to sequence regions of high GC content.

Double-stranded DNA sequencing was performed by a modification of the protocol of Wang et al. (37) with double-stranded plasmid DNA prepared by using a Promega Magic Miniprep spin column (Promega, Madison, Wis.) as the template. The entire sequence of both strands was determined.

In vitro transcription-translation. In vitro transcription-translation with an *E. coli* S30 coupled transcription-translation system (Promega) was done by a modification of a protocol described elsewhere (26b). Synthesized proteins were precipitated with acetone and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Pulse labelling. An overnight culture of *S. marcescens* cells was diluted in minimal medium (1:100) and grown at 30°C to an optical density at 600 nm of 1.0 to 1.5. When induction was needed, the cells were induced with 1 mM IPTG for 15 min at this point. To 1 ml of culture in a snap cap tube, 3 μl of [^{35}S]Met was added. After a 2-min pulse, 100 μl of cold L-Met (100 mg/ml) was added. A 0.3-ml volume of culture was transferred to an Eppendorf tube on ice containing 33 μl of KCN. The remainder of the culture was returned to the 30°C shaker until the 30- and 60-min time points. At each time point, 0.3 ml of culture was transferred to another cold Eppendorf tube containing KCN.

The cells were centrifuged at 13,000 rpm (Sorvall centrifuge, rotor SS34) for 10 min, the supernatant was transferred to a screw-cap tube, and 2.5 volumes of cold acetone was added. The tube was left on ice for 15 min to precipitate the proteins, and then it was centrifuged at 13,000 rpm for 10 min at 4°C. The pellet was washed with 1 ml of 70% ethanol, dried under a vacuum, and resuspended in 100 μl of SDS sample buffer.

The cell pellet was resuspended in 1 ml of cold wash buffer (150 mM NaCl, 50 mM Tris-HCl [pH 8.0]), centrifuged, washed again with 0.5 ml of cold wash buffer, centrifuged again, and resuspended in 100 μl of SDS sample buffer. The samples were heated to denature proteins (70°C, 15 min). Next, 5 μl of cell fraction and 20 μl of supernatant fraction sample were loaded on an SDS-polyacrylamide gel. The gels were exposed to X-Omat film for detection of the labelled protein bands.

Purification of lipase. Plasmid pSE420-LipRB was amplified by addition of 100 μg of chloramphenicol per ml to growing cells. Cells were then washed once with fresh LB and allowed to grow overnight, and lipase production was induced by addition of 1 mM IPTG. After 4 h, the culture supernatant was harvested by centrifugation at 10,000 rpm at 4°C for 1 h. The supernatant was then sterilized by filtration (0.2-μm-pore-size filter; Millipore), and 0.02% NaN_3 was added. The volume of the supernatant was reduced by ultrafiltration (Amicon YM 30 membrane; 30-kDa cutoff) to 100 ml, washed with thrice the volume of 50 mM Tris-HCl (pH 8.0), and further reduced to 10 ml.

The following purification steps were performed at room temperature. Anion-exchange chromatography was performed with a MonoQ HR 5/5 column (Pharmacia, Freiburg, Germany). A 5-ml volume of concentrated supernatant was applied to the column, which was previously equilibrated with 50 mM Tris-HCl (pH 8.0). Unbound protein was washed out with 5 ml of buffer. Proteins were then eluted with 0 to 1 M NaCl–50 mM Tris-HCl (pH 8.0) in a 30-ml linear gradient. Protein was detected by measuring A_{280}. The fractions (1 ml) were assayed for lipase activity by the p-Npp method. Hydrophobic interaction chromatography (HIC) was with butyl-Sepharose CL (Pharmacia). The gel (10 ml) was washed twice with 4 M NaCl–50 mM Tris-HCl (pH 8.0) and poured into a column (1-cm diameter), and the gel bed was washed with 30 ml of the initial buffer. The combined peak fractions from the anion-exchange chromatography received solid NaCl to a final concentration of 4 M. The material was applied to the column, and unbound protein was washed out with 10 ml of the initial buffer. Proteins were then eluted with 0 to 4 M NaCl–50 mM Tris-HCl (pH 8.0) in a 30-ml linear gradient. Protein was detected by measuring A_{280}. The fractions (1 ml) were then assayed for lipase activity by the p-Npp method.

Protein gel electrophoresis. SDS-PAGE gels were made with 10 to 15% polyacrylamide by using the standard discontinuous system of Laemmli (21). Samples were loaded in sample buffer containing 4% (wt/vol) SDS. Gels were stained according to the method of Blum et al. (4). Isoelectric focusing was done as described previously (15).

RESULTS

Cloning and sequencing of the lipase gene. A genomic library of *S. marcescens* SM6 had previously been constructed in the phage vector λEMBL3 (2). This library was screened on rhodamine B-olive oil indicator plates for lipase activity, and four positive plaques were identified. A 2.6-kb *Sal*I fragment present in all four clones was subcloned into the *Sal*I site of plasmid pBGS18 to make pLipS1, thereby conferring on *E. coli* a lipase-positive phenotype, and subsequently moved into the *Sal*I site of pUC119 for sequencing. The complete nucleotide sequence is shown in Fig. 1. The total length of the fragment is 2,643 nt, and it has a GC content of 60%, corresponding to the codon usage described for *Serratia* spp. (14).

In order to locate the start and stop codons of the open reading frame (ORF), ExoIII deletion clones of the fragment were analyzed for lipase production. Two of these deletions

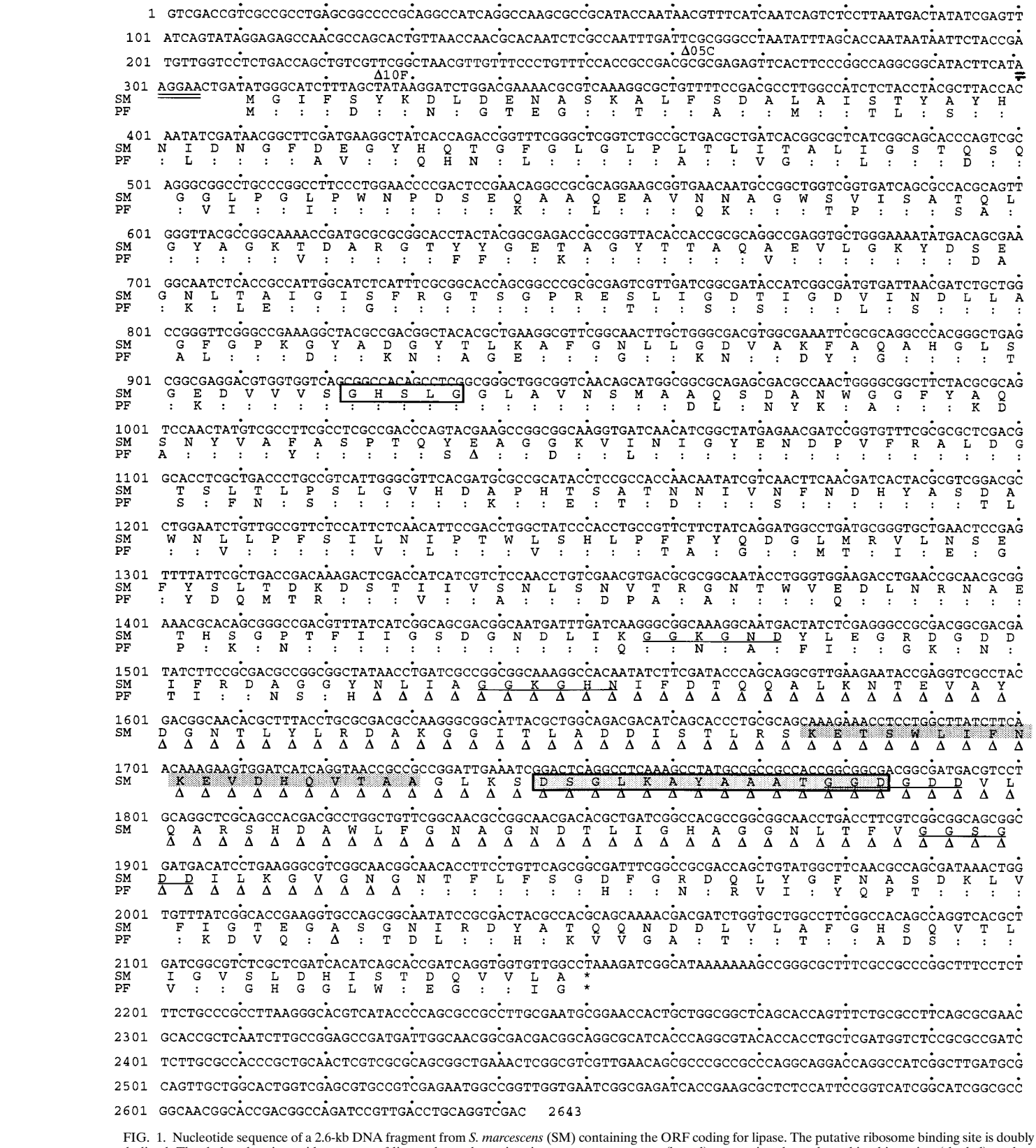

FIG. 1. Nucleotide sequence of a 2.6-kb DNA fragment from *S. marcescens* (SM) containing the ORF coding for lipase. The putative ribosome binding site is doubly underlined. The deduced amino acid sequence of lipase shows the active-site consensus sequence (boxed), a putative charged amphipathic region (shaded), and an aspartate box (boxed and shaded). Glycine-rich consensus motifs are underlined. The end points of the two deletions, Δ05C and Δ10F, which define the gene start are shown above the sequence. The related sequence from *P. fluorescens* (PM) is shown below the SM amino acid sequence. Identical residues are shown by colons, changes are indicated, and residues missing in one sequence are designated Δ. The sequence data appear in GenBank under the accession number U11258.

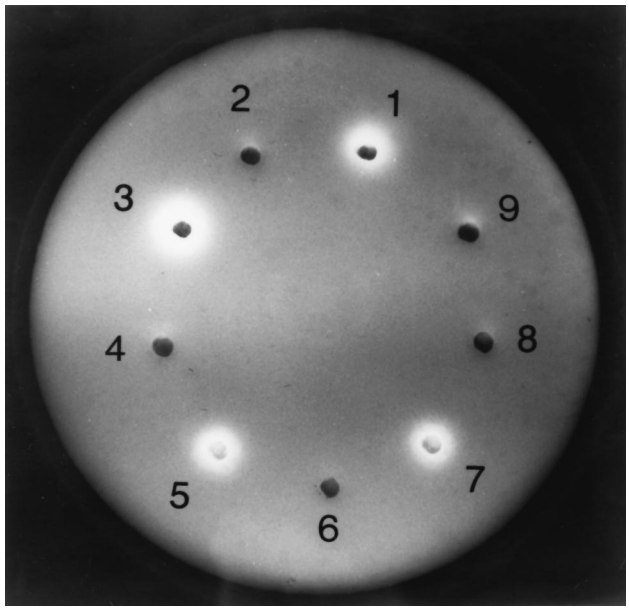

FIG. 2. Rhodamine B-triolein agar plate showing (1) *S. marcescens* SM6, (2) *E. coli* JM101(pGSD6), (3) *E. coli* JM101(pGSD6, pSE420-LipRB), (4) *E. coli* JM101(pSE420-LipRB), (5) *E. coli* JM101(pRSC6, pSE420-LipRB), (6) *E. coli* JM101(pRSC6), (7) *E. coli* K-12(pHyl152, pSE420-LipRB), (8) *E. coli* K-12(pHyl152), and (9) *E. coli* JM101. A 5-μl volume of culture supernatant (sterile filtered) was poured into each preformed hole, and the plate was incubated at 30°C overnight. Halos around the holes (strains labelled 1, 3, 5, and 7) indicate lipase production.

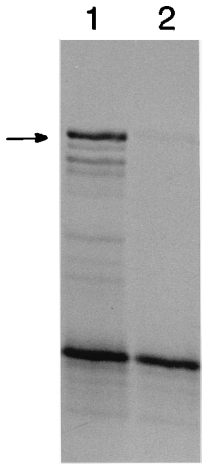

FIG. 3. In vitro transcription-translation of pBGS18-Lip05C (lane 1) and pBGS18-Lip10F (lane 2) in the presence of 2 mg of phenylmethylsulfonyl fluoride ml^{-1} by using the *E. coli* S30 system. The ^{35}S-labeled proteins were separated by SDS-PAGE and visualized by autoradiography (see Materials and Methods). The 64-kDa protein band is marked with an arrow.

spanned the translational start codon for lipase: Lip05C has 260 nt removed from the upstream *Sal*I site and carried only 50 nt upstream from the ATG start codon, whereas Lip10F had an additional 65 nt deleted (15 nt beyond the ATG start). The former remained Lip^+, whereas the latter was Lip^-. This allowed us to place the start codon at nt 311 and the stop codon at nt 2150, creating an ORF of 1,842 nt encoding a protein of 613 amino acids. A Shine-Dalgarno sequence, AAGGAA, was found 7 bases upstream of the ATG start codon.

Analysis of the ORF did not indicate the presence of a secretion signal sequence at the N terminus of the lipase or the existence of transmembrane helices. The amino acid sequence derived from the nucleotide sequence contains the lipase active-site consensus sequence Gly-X_1-Ser-X_2-Gly, which is found in the majority of bacterial and eucaryotic lipases. The predicted protein has an M_r of 64,800 and a pI of 4.3 and contains no cysteine residues.

Lipase is secreted by the class I secretion pathway. When plasmid pGSD6 (7), carrying the protease secretion genes of *Erwinia chrysanthemi* which are homologous to the *E. coli* α-hemolysin secretion genes *hylB*, *hylD*, and *tolC* (13), was present, lipase was released by *E. coli* into the extracellular medium. In Fig. 2, lipase activity found in culture supernatants is shown. Similar experiments using the excretion functions from an α-hemolytic strain of *E. coli* showed that the C-terminal excretion signal is recognized by the *E. coli* transport functions, allowing lipase to be released into the culture supernatant (Fig. 2).

A glycine-rich repeated motif L-X-G-G-B-G-B-B-X, present in members of the RTX toxin family and in proteins secreted by the class I secretion pathway, was also found repeated four times, although with slight modifications, in the sequence of the *S. marcescens* lipase. In addition, we identified an amphipathic charged region ranging from Lys-456 to Ala-474 followed by a so-called aspartate box from Asp-478 to Asp-492 (Fig. 1), which is defined as a stretch of 12 to 14 mainly small and uncharged residues rich in Ala and Ser, flanked by positively charged residues (usually Asp) (19). A hydroxylated tail at the extreme C terminus of the protein is missing. Our results indicated that this lipase is secreted without being processed in a single step via the class I secretion pathway.

In vitro expression. In order to verify the size of the translated protein predicted by the ORF, in vitro transcription-translation was performed with plasmid pBGS18-Lip05C. A lipase-negative clone, pBGS18-Lip10F, was used as a negative control in the experiment. Figure 3 shows the predominant band at about 64 kDa, agreeing well with the molecular mass of 64.8 kDa predicted from the nucleotide sequence. The spectrum of bands with lower molecular weight is likely due to proteolytic degradation of the polypeptide. Repeating the experiment in the absence of protease-inhibiting phenylmethylsulfonyl fluoride greatly increased the intensity of these bands. The dark band migrating at the bottom of the gels is neomycin phosphotransferase encoded by pBGS18.

Expression in *E. coli*. Lipase activity in *E. coli* when expressed from pBGS18, only reached *S. marcescens* wild-type level. As more protein was needed for the purification, the gene was subcloned into the expression vector pSE420 (5), providing a strong *trc* promoter which can be repressed by the *lac* repressor and induced with IPTG. Additionally, the vector contains the bacteriophage T7 gene 10 translational enhancer, an upstream minicistron for efficient translational restart, and ribosome binding sites flanked by A/T-rich regions. Furthermore, the transcriptional antitermination region from the *E. coli rrnB* rRNA operon is located downstream from the *trc* promoter, which may facilitate transcription through highly structured areas of the recombinant mRNA (5). However, the level of lipase activity was still very low.

To test if the low-level lipase activity was a result of post-translational degradation or inefficient translation, a *lipA-lacZ* gene fusion was constructed with pSE420-Lip05C and pUC118-Lip05C. β-Galactosidase is known to be stable, so the enhanced transcriptional and translational efficiency of pSE420 should lead to a higher level of β-galactosidase activity than obtained with pUC18. This result was not observed (data

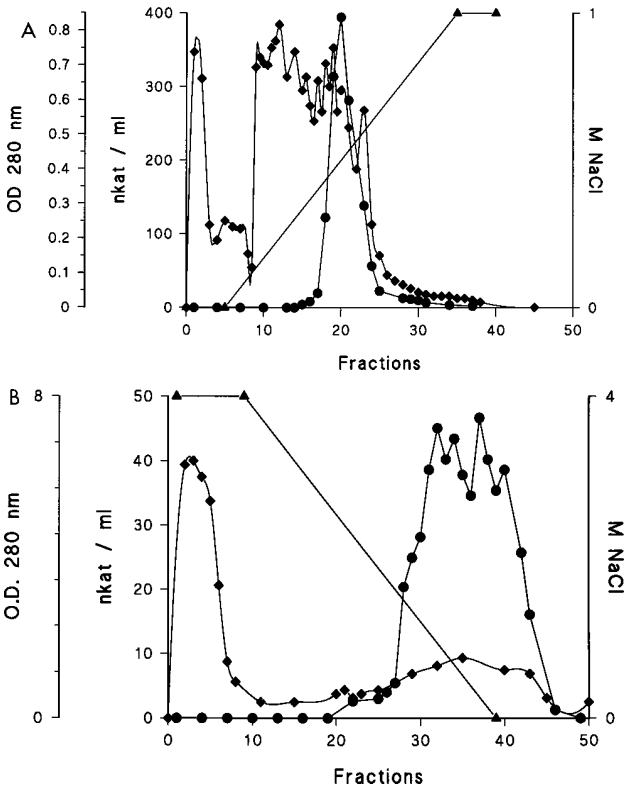

FIG. 4. Column chromatographic purification of *S. marcescens* lipase isolated from *E. coli* culture supernatant. Activity and absorbance were measured for each fraction. (A) Anion-exchange chromatography was performed with a MonoQ column, and proteins were eluted in a 30-ml gradient with increasing concentrations of NaCl in 50 mM Tris-hydrochloride buffer (pH 8.0). (B) HIC chromatography was performed with a butyl-Sepharose column, and proteins were eluted in the buffer described in the legend to panel A with decreasing concentrations of NaCl. Lipase activity was assayed with *p*-nitrophenyl palmitate as a substrate. ●, lipase activity (nanokatals per milliliter); ▲, gradient (NaCl concentration); ◆, optical density at 280 nm (O.D. 280 nm).

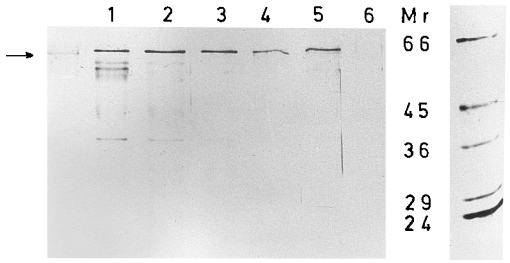

FIG. 5. SDS-PAGE analysis of fractions obtained from column chromatography. The gel was stained with silver (4). Lanes 1 to 6 correspond to fractions 37, 33, 32, 31, 30, and 16, respectively, shown in Fig. 4B. M_rs (in thousands) of marker proteins are indicated. The position of the putative *S. marcescens* lipase (64 kDa) is marked with an arrow.

not shown). Therefore, the low level of lipase activity was not due to instability but likely resulted from inefficient translation.

The lipase gene was then PCR amplified to introduce an *Nco*I site preceding the start codon of the ORF and cloned into the *Nco*I site of plasmid pSE420, thus eliminating the ribosomal binding sequence of the lipase gene and substituting the optimized ribosomal binding sequence of pSE420 with its adjacent translational enhancer. This was expected to improve translational efficiency. The level of lipase expression, although higher than obtained with previous plasmids, again did not significantly exceed the wild-type level. However, amplification of the vector pSE420-LipRB with chloramphenicol in situ led to a fivefold-higher expression of lipase, which was finally sufficient for purification.

Purification of the lipase protein. Recombinant lipase was purified from the extracellular medium by anion-exchange chromatography and HIC as described in Materials and Methods. Anion-exchange chromatography yielded a relatively sharp peak of lipase activity eluting at an NaCl concentration of about 0.5 M (Fig. 4A). However, SDS-PAGE analysis of these samples revealed the presence of at least five different protein bands. Therefore, the lipase-containing fractions were pooled and Sepharose gels with octyl and butyl ligands were used for HIC. Lipase was tightly bound to octyl-Sepharose and did not elute with either 10% (vol/vol) isopropanol or 1% (wt/vol) Na-deoxycholate in 50 mM Tris-hydrochloride (pH 8.0) at room temperature. Decreasing the hydrophobic interactions by reduction of the temperature to 4°C had no effect. Only when octyl-Sepharose was replaced with butyl-Sepharose could lipase be eluted by application of a linear gradient of NaCl (Fig. 4B). Subsequent analysis of the peak fractions by SDS-PAGE showed a single band migrating at about 63 kDa (Fig. 5). We presume this band is lipase, but there was insufficient material to perform N-terminal sequencing for confirmation. This M_r corresponded well with the results of the genetic analysis and the in vitro expression. Assuming the band is in fact lipase, this result demonstrated that no significant processing of the protein had occurred, as would be expected for proteins that use the class I secretion pathway.

Biochemical properties. *S. marcescens* lipase was found to be associated with LPS by application of an LPS-specific silver-staining method after SDS-PAGE analysis of culture supernatants. These high-M_r lipase-LPS micelles could not be solubilized without significant loss of enzymatic activity, thereby preventing purification of the lipase (26a). In *E. coli*, high-M_r aggregates were observed to be present in the culture supernatant upon gel chromatography on Sepharose 6B columns (data not shown). These aggregates could be disintegrated at least partially by solubilization with the zwitterionic detergent CHAPS {3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate}, suggesting that LPS might be associated with the lipase in *E. coli* as well, although the total amount was too small to be detected by silver staining of SDS-PAGE gels.

Isoelectric focusing revealed a pI of 5.8, which did not fit precisely the pI of 4.3 predicted from the sequence. A broad pH optimum ranging from pH 6 to 10 was observed. A detailed analysis of substrate and positional specificity has not yet been undertaken. Nevertheless, we observed by titrimetric and plate assays that this lipase was able to hydrolyze long-chain triglyceride esters, thereby allowing its classification as a true lipase (EC 3.1.1.3).

DISCUSSION

The lipase gene from *S. marcescens* SM6 has been cloned from a genomic library by screening plaques for lipase released by cell lysis on lipase indicator plates. A 2.6-kb *Sal*I fragment bearing the lipase gene has been sequenced, and an ORF encoding the lipase was found. The nucleotide sequence of the *S. marcescens* lipase gene is 1,842 nt long and encodes a protein of 613 amino acids with a predicted molecular mass of 64.8 kDa. This lipase is larger than the lipases of the *Pseudomonas* group of gram-negative bacteria, but lipases of *Aeromonas*

hydrophila and *Xhenorhabdus luminescens* are even larger (see reference 16 for a comparison of different lipases). The pI of this lipase is 4.3, which is lower than the pI determined experimentally (pI of 5.8). We assume that the latter value is closer to reality; however, the isoelectric focusing experiments were performed with limited amounts of pure lipase protein.

The lipases from *P. fluorescens* strains (17, 34) show remarkable sequence similarity to that of *S. marcescens*, with an identity of about 65% over virtually the entire length of the sequence. However, the *P. fluorescens* lipase is about 450 amino acids in length whereas the *S. marcescens* enzyme is significantly longer, at 614 residues. Alignment of the protein sequences shows the *S. marcescens* enzyme to differ from the *P. fluorescens* sequence by having a single large insertion about 160 residues from its carboxyl terminus.

There are no cysteine residues in the deduced amino acid sequence of lipase. Cysteines are often involved in the formation of disulfide bonds in proteins, and proteins without cysteines are generally more flexible because of the lack of disulfide bonds. It has been noted that many extracellular bacterial proteins have few or no cysteines, a characteristic that may be important for some mechanisms of extracellular secretion. Another possible advantage of the lack of disulfides in lipase is that it may more readily allow the conformational change that accompanies interfacial activation.

A comparison of the amino acid sequence of *S. marcescens* lipase and those from other bacterial, mammalian, and fungal lipases reveals little primary sequence similarity except for the common conserved Gly-X_1-Ser-X_2-Gly active-center sequence, where X_1 is histidine in bacterial lipases. The serine is presumed to be the essential active-site residue, necessary for catalytic activity.

The surprisingly large size of the *Serratia* lipase warranted verification of the sequence by demonstrating that a protein of this size was actually coded for and produced by the gene identified. Because of the high GC content of *S. marcescens*, sequencing errors are common. In vitro transcription-translation of the cloned *S. marcescens* gene with an extract derived from *E. coli* demonstrated production of a protein of about 65 kDa requiring an intact lipase gene template. In vivo pulse-labelling experiments also demonstrated lipase gene-dependent production of a protein band of between 63 and 65 kDa. Both values agree well with the molecular mass of 64.8 kDa predicted from the DNA sequence. Additionally, both in vivo- and in vitro-synthesized enzymes were enzymatically active, indicating that lipase has catalytic activity as soon as it is synthesized and that no modification, processing, or accessory proteins are required for the activity of this enzyme.

The *S. marcescens* lipase is distinct from other bacterial lipases in that it lacks an N-terminal signal peptide. Only the *P. fluorescens* lipase (9) also lacks such a signal. Both lipases contain several copies of a glycine-rich motif, L-X-G-G-B-G-B-B-X, that is found in RTX toxins (3, 38) and related proteins which utilize the ABC transporter system (11). Although this motif is not essential for secretion, since the C-terminal region alone has been shown to be both necessary and sufficient (19), it is strongly predictive for secretion with the ABC-type system. Another protein from *S. marcescens*, the extracellular metalloprotease which is related to a similar protease from *Erwinia* species, also carries these motifs and has been shown to use the same secretory system (8, 12, 33).

ACKNOWLEDGMENTS

This work was supported in part by grant GM36891 from the National Institutes of Health, grant 26521178 from the Texas Advanced Research Program, and a grant from the University of Houston to M.J.B. National Science Foundation grant DIR 9109294 provided some equipment used in this work.

ADDENDUM

During the preparation of this paper, the nucleotide sequence of a lipase from *S. marcescens* Sr41 was published (1). However, secretion of this lipase by the class I secretion pathway, or by *E. coli*, was not demonstrated.

REFERENCES

1. **Akatsuka, H., E. Kawai, K. Omori, S. Komatsubara, T. Shibatani, and T. Tosa.** 1994. The *lipA* gene of *Serratia marcescens* which encodes an extracellular lipase having no N-terminal signal peptide. J. Bacteriol. **176:**1949–1956.
2. **Ball, T. K., P. N. Saurugger, and M. J. Benedik.** 1987. The extracellular nuclease gene of *Serratia marcescens* and its secretion from *Escherichia coli*. Gene **57:**183–192.
3. **Blight, M. A., and I. B. Holland.** 1990. Structure and function of haemolysin B, P-glycoprotein and other members of a novel family of membrane translocators. Mol. Microbiol. **4:**873–880.
4. **Blum, H., B. Hildburg, and H. Gross.** 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis **8:**93–99.
5. **Brosius, J.** 1992. Compilation of superlinker vectors. Methods Enzymol. **216:**469–483.
6. **Coote, J. G.** 1992. Structural and functional relationships among the RTX toxin determinants of gram-negative bacteria. FEMS Microbiol. Rev. **88:** 137–162.
7. **Dahler, G. S., F. Barras, and N. T. Keen.** 1990. Cloning of genes encoding extracellular metalloproteases from *Erwinia chrysanthemi* EC16. J. Bacteriol. **172:**5803–5815.
8. **Delepelaire, P., and C. Wandersman.** 1989. Protease secretion by *Erwinia chrysanthemi*: proteases B and C are synthesized and secreted as zymogens without a signal peptide. J. Biol. Chem. **264:**9083–9089.
9. **Duong, F., C. Soscia, A. Lazdunski, and M. Murgier.** 1994. The *Pseudomonas fluorescens* lipase has a C-terminal secretion signal and is secreted by a three-component bacterial ABC-exporter system. Mol. Microbiol. **11:**1117–1126.
10. **Falkow, S., J. Marmur, W. F. Carey, W. M. Spilman, and L. S. Baron.** 1961. Episomic transfer between *Salmonella typhosa* and *Serratia marcescens*. Genetics **46:**703–706.
11. **Fath, M. J., and R. Kolter.** 1993. ABC transporters: bacterial exporters. Microbiol. Rev. **57:**995–1017.
12. **Ghigo, J.-M., and C. Wandersman.** 1992. Cloning, nucleotide sequencing and characterization of the gene encoding *Erwinia chrysanthemi* B374 PrtA metalloprotease: a third metalloprotease secreted via a C-terminal secretion signal. Mol. Gen. Genet. **236:**135–144.
13. **Gray, L., K. Baker, B. Kenny, N. Mackman, R. Haigh, and I. B. Holland.** 1989. A novel C-terminal signal sequence targets *Escherichia coli* haemolysin directly to the medium. J. Cell. Sci. Suppl. **11:**45–57.
14. **Grimont, P. A. D., and F. Grimont.** 1978. The genus *Serratia*. Annu. Rev. Microbiol. **32:**221–248.
15. **Jaeger, K.-E., F.-J. Adrian, H. E. Meyer, R. E. W. Hancock, and U. K. Winkler.** 1992. Extracellular lipase from *Pseudomonas aeruginosa* is an amphiphilic protein. Biochim. Biophys. Acta **1120:**315–321.
16. **Jaeger, K.-E., S. Ransac, B. W. Dijkstra, C. Colson, M. van Heuvel, and O. Misset.** 1994. Bacterial lipases. FEMS Microbiol. Rev. **15:**29–63.
17. **Johnson, L. A., I. R. Beacham, I. C. MacRae, and M. L. Free.** 1992. Degradation of triglycerides by a pseudomonad isolated from milk: molecular analysis of a lipase-encoding gene and its expression in *Escherichia coli*. Appl. Environ. Microbiol. **58:**1776–1779.
18. **Kenny, B., C. Chervaux, and B. I. Holland.** 1994. Evidence that residues −15 to −46 of the haemolysin secretion signal are involved in early steps of secretion, leading to recognition of the translocator. Mol. Microbiol. **11:**99–109.
19. **Kenny, B., S. Taylor, and I. B. Holland.** 1992. Identification of individual amino acids required for secretion within the haemolysin HlyA. C-terminal targeting region. Mol. Microbiol. **6:**1477–1489.
20. **Kouker, G., and K.-E. Jaeger.** 1987. Specific and sensitive plate assay for bacterial lipases. Appl. Environ. Microbiol. **53:**211–213.
21. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
22. **Létoffé, S., P. Delepelaire, and C. Wandersman.** 1991. Cloning and expression in *Escherichia coli* of the *Serratia marcescens* metalloprotease gene: secretion of the protease from *E. coli* in the presence of the *Erwinia chrysanthemi* protease secretion functions. J. Bacteriol. **173:**2160–2166.
23. **Létoffé, S., and C. Wandersman.** 1992. Secretion of CyaA-PrtB and HylA-PrtB fusion proteins in *Escherichia coli*: involvement of the glycine-rich repeat domain of *Erwinia chrysanthemi* protease B. J. Bacteriol. **174:**4920–4927.
24. **Neidhardt, F. C., P. L. Bloch, and D. F. Smith.** 1974. Culture medium for

enterobacteria. J. Bacteriol. **119:**736–747.
24a. **Neumann, S., and U. K. Winkler.** Unpublished data.
25. **Noegel, A., U. Rdest, W. Springer, and W. Goebel.** 1979. Plasmid cistrons controlling synthesis and excretion of the exotoxin α-haemolysin of *Escherichia coli*. Mol. Gen. Genet. **175:**343–350.
26. **Palleroni, N. J.** 1993. *Pseudomonas* classification. Antonie van Leeuwenhoek J. Microbiol. Serol. **64:**231–251.
26a. **Proft, D., and K. E. Jaeger.** Unpublished data.
26b. **Promega.** 1991. Protocols and applications guide, 2nd ed. Promega, Madison, Wis.
27. **Pugsley, A. P.** 1993. The complete general secretory pathway in gram-negative bacteria. Microbiol. Rev. **57:**50–108.
28. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
29. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
30. **Schulein, R.** 1993. Ph.D. thesis. University of Würzburg, Würzburg, Germany.
31. **Spratt, B. G., P. J. Hedge, S. te Heesen, A. Edelman, and J. K. Broome-Smith.** 1986. Kanamycin-resistant vectors that are analogues of plasmids pUC8, pUC9, pEMBL8 and pEMBL9. Gene **41:**337–342.
32. **Stuer, W., K.-E. Jaeger, and U. K. Winkler.** 1986. Purification of extracellular lipase from *Pseudomonas aeruginosa*. J. Bacteriol. **168:**1070–1074.
33. **Suh, Y., and M. J. Benedik.** 1992. Production of active *Serratia marcescens* metalloprotease from *Escherichia coli* by α-hemolysin HylB and HylD. J. Bacteriol. **174:**2361–2366.
34. **Tan, Y., and K. J. Miller.** 1992. Cloning, expression, and nucleotide sequence of a lipase gene from *Pseudomonas fluorescens* B52. Appl. Environ. Microbiol. **58:**1402–1407.
35. **Tommassen, J., A. Filloux, M. Bally, M. Murgier, and A. Lazdunski.** 1992. Protein secretion in *Pseudomonas aeruginosa*. FEMS Microbiol. Rev. **103:** 73–90.
36. **Wandersman, C.** 1992. Secretion across the bacterial outer membrane. Trends Genet. **8:**4776–4780.
37. **Wang, L. M., D. K. Weber, T. Johnson, and A. Y. Sakaguchi.** 1988. Double-stranded DNA sequencing with T7 polymerase. BioTechniques **6:**843–845.
38. **Welch, R. A.** 1991. Pore-forming cytolysins of gram-negative bacteria. Mol. Microbiol. **5:**521–528.
39. **Winkler, U., K. B. Heller, and B. Folle.** 1978. Pleiotropic consequences of mutations towards antibiotic-hypersensitivity in *Serratia marcescens*. Arch. Microbiol. **116:**259–268.
40. **Winkler, U. K., and M. Stuckmann.** 1979. Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marcescens*. J. Bacteriol. **138:**663–670.
41. **Wohlfarth, S., C. Hoesche, C. Strunk, and U. K. Winkler.** 1992. Molecular genetics of the extracellular lipase of *Pseudomonas aeruginosa* PAO1. J. Gen. Microbiol. **138:**1325–1335.