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A Novel Lipolytic Enzyme Located in the Outer Membrane of *Pseudomonas aeruginosa*

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A lipase-negative deletion mutant of *Pseudomonas aeruginosa* PAO1 still showed extracellular lipolytic activity toward short-chain *p*-nitrophenylesters. By screening a genomic DNA library of *P. aeruginosa* PAO1, an esterase gene, *estA*, was identified, cloned, and sequenced, revealing an open reading frame of 1,941 bp. The product of *estA* is a 69.5-kDa protein, which is probably processed by removal of an N-terminal signal peptide to yield a 67-kDa mature protein. A molecular mass of 66 kDa was determined for ^{35}S-labeled EstA by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. The amino acid sequence of EstA indicated that the esterase is a member of a novel GDSL family of lipolytic enzymes. The *estA* gene showed high similarity to an open reading frame of unknown function located in the *trpE-trpG* region of *P. putida* and to a gene encoding an outer membrane esterase of *Salmonella typhimurium*. Amino acid sequence alignments led us to predict that this esterase is an autotransporter protein which possesses a carboxy-terminal β-barrel domain, allowing the secretion of the amino-terminal passenger domain harboring the catalytic activity. Expression of *estA* in *P. aeruginosa* and *Escherichia coli* and subsequent cell fractionation revealed that the enzyme was associated with the cellular membranes. Trypsin treatment of whole cells released a significant amount of esterase, indicating that the enzyme was located in the outer membrane with the catalytic domain exposed to the surface. To our knowledge, this esterase is unique in that it exemplifies in *P. aeruginosa* (i) the first enzyme identified in the outer membrane and (ii) the first example of a type IV secretion mechanism.

Pseudomonas aeruginosa is a gram-negative soil bacterium that is also well known to be an important opportunistic human pathogen, which secretes a variety of proteins into the extracellular medium. Three of these are lipolytic enzymes: two extracellular phospholipases C (PLC) and a lipase (20, 53). Apart from the phospholipases (EC 3.1.4.3), the term "lipolytic enzymes" comprises lipases (EC 3.1.1.3) and esterases (EC 3.1.1.1), which hydrolyze glycerol esters of both short- and long-chain fatty acids. Lipases are, by definition, carboxylesterases that have the ability to hydrolyze long-chain acylglycerols ($\geq C_{10}$), whereas esterases hydrolyze ester substrates with short-chain fatty acids ($\leq C_{10}$) (57). However, it should be emphasized that lipases are perfectly capable of hydrolyzing esterase substrates. In *P. aeruginosa*, the phospholipases PLC-H (heat-labile hemolysin) and PLC-N (nonhemolytic) have molecular masses of 78,000 and 73,000, respectively (56), and hydrolyze a variety phosphoric monoester substrates. The lipase LipA has a molecular mass of 29,000 and hydrolyzes water-insoluble carboxylic esters of long-chain fatty acids, e.g., trioleoyl glycerol and *p*-nitrophenylpalmitate (53). In addition to these secreted enzymes, an esterase tightly bound to the outer membrane of *P. aeruginosa* which has a molecular mass of 55,000 and preferentially hydrolyzes long-chain acyl thio- or oxyesters has been described (37).

There are two main reasons to study lipolytic enzymes of *P. aeruginosa*: (i) their important role as virulence factors and (ii) their biotechnological potential.

Clinical *P. aeruginosa* strains isolated from cystic fibrosis patients produce both lipase and PLC (21). A synergistic effect of PLC-H and LipA which led to the complete hydrolysis in vitro of the major lung surfactant lipid dipalmitoylphosphatidyl-choline has been demonstrated (20). Furthermore, these enzymes induce the release of the inflammatory mediator 12-hydroxyeicosatetraenoic acid from human platelets (27). These findings suggest that the lipolytic enzymes of *P. aeruginosa* act as virulence factors. The outer membrane-bound esterase may enable *P. aeruginosa* to utilize a variety of acyl esters as carbon sources; however, its role in pathogenicity has not been studied (37).

Lipases also play an important role in a variety of biotechnological applications (23). This potential is based on their ability to catalyze not only the hydrolysis of triglycerides but also their synthesis from glycerol and fatty acids, which may proceed with high specificity and enantioselectivity (24). In particular, *P. aeruginosa* lipase catalyzes the stereoselective conversion of a variety of amines as well as primary and secondary alcohols (25). Recently, this lipase was used to demonstrate the principle of creating a biocatalyst with high enantioselectivity toward a given substrate by applying the technique of directed evolution (41).

In the culture supernatant of the lipase-negative deletion mutant *P. aeruginosa* PABS1, we detected residual lipolytic activity, which led us to identify a novel esterase. The corresponding gene was cloned and expressed, and the encoded protein was analyzed with respect to its cellular location.

MATERIALS AND METHODS

Strains and plasmids. The strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* PAO1 and PABS1 were used throughout this study. *Escherichia coli* JM109 was used as a host for cloning, *E. coli* S17-1 was used for conjugational transfer of mobilizable plasmids, and *E. coli* BL21(DE3)(pLysS) (Novagene) was used for selective expression of plasmid-encoded esterase.

Media and growth conditions. Bacteria were grown in glass tubes overnight at 37°C, used to inoculate 5 ml of fresh medium to an optical density at 580 nm (OD_{580}) of 0.05, and grown for 24 h under aeration. *P. aeruginosa* was grown in nutrient broth (Oxoid), supplemented when necessary with 100 μg of tetracycline per ml, 300 μg of chloramphenicol per ml, or 500 μg of carbenicillin per ml. *E.*

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
P. aeruginosa PAO1	wild-type	19
P. aeruginosa PABS1	Δ*lipA* Δ*lipH*	45
P. aeruginosa 2B18	*pilD(xcpA)*::Tn*5*	49
P. aeruginosa PUS13	Δ*xcpQ*	1
E. coli JM109	F′ *traD36 lacI*^{q} Δ(*lacZ*)*M15 proA*$^+$*B*$^+$/*el4*(McrA$^-$) Δ(*lac-proAB*) *thi gyrA96* (Nal^r) *endA1 hsdR17* (r_K^- m_K^+) *relA1 supE44 recA1*	65
E. coli S17-1	*thi pro hsdRM*$^+$, chromosomally integrated [RP4-2-Tc::Mu:Km^r::Tn*7*, Tra$^+$ Tri^r Str^r]	46
E. coli BL21(DE3)(pLysS)	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (λ*cIts857 ind1 Sam7 nin5 lacUV5-T7* gene1) [pLysS Cm^r T7-Lysozyme]	51, 52
Plasmids		
pLAFR3	Cos sites Tc^r P_{lac} mob	47
pUCPKS/SK	Amp^r *lacZ*α P_{lac} P_{T7}	60
pBBR1MCS	Cm^r mob *lacZ*α P_{lac}	29
pLAFR3-21.P	pLAFR3 containing a 22.1-kb fragment of chromosomal DNA of *P. aeruginosa* PAO1, including *estA*	This study
pSKX+	pUCPSK containing a 3.3-kb *Xho*I DNA fragment of *P. aeruginosa*, including *estA*, under control of P_{lac}	This study
pSKX−	As pSKX+, but *estA* in the opposite orientation under control of P_{T7}	This study
pBBX+	pBBR1MCS containing a 3.3-kb *Xho*I DNA fragment of *P. aeruginosa*, including *estA*, under control of P_{lac}	This study
pLip1	pUCPSK containing a 2.85-kb fragment carrying the *lipA-lipH* lipase operon	Unpublished data

coli was grown in Luria broth (LB) medium or M9 minimal medium (42), supplemented when necessary with 25 μg of tetracycline per ml, 100 μg of ampicillin per ml, or 50 μg of chloramphenicol per ml.

General DNA manipulations. Plasmid DNA was prepared as described by Birnboim and Doly (5) and purified by anion-exchange chromatography on Qia-tips (Qiagen). Chromosomal DNA was prepared as described by Gamper et al. (15). Recombinant DNA techniques were performed essentially as described by Sambrook et al. (42). Restriction endonuclease reactions and bacteriophage T4 DNA ligase treatments were done as recommended by the manufacturers. DNA fragments were analyzed on 0.4 to 1% (wt/vol) agarose gels.

Construction of a genomic library. A genomic library of *P. aeruginosa* PAO1 chromosomal DNA in cosmid pLAFR3 was constructed as described by Visca et al. (58). Chromosomal DNA was partially digested with *Sau*3A to obtain fragments of >15 kb. Cosmid DNA was linearized with either *Eco*RI or *Hin*dIII, dephosphorylated, mixed, and digested with *Bam*HI. Chromosomal DNA fragments were added and ligated. The λ-DNA in vitro packaging module (Amersham) was used for DNA packaging and infection of *E. coli* S17-1. The genomic library consisted of 15,000 individual clones each carrying an insert of 21 to 28 kb (average size), statistically representing 99.9% of the *P. aeruginosa* genome.

Screening of the genomic library. The individual clones were screened in *P. aeruginosa* PABS1 on esterase indicator plates. The library clones were conjugated from *E. coli* S17-1 into *P. aeruginosa* PABS1 and plated on esterase indicator plates to detect the formation of halos.

Cell fractionation. Cultures (volume, 100 ml) of *P. aeruginosa* and *E. coli* grown in LB medium for 24 h were separated by centrifugation (8,000 × *g* for 5 min) into cells and culture supernatant, which was used to determine the extracellular enzyme activity. The cells were resuspended in 20 ml of 100 mM Tris-HCl (pH 8.0) and disrupted by two passages through a French press. Cell debris was removed by centrifugation (5,000 × *g* for 15 min), and the supernatant was subjected to ultracentrifugation at 100,000 × *g* for 2 h to collect the crude membrane fraction, which was resuspended in 5 ml of 100 mM potassium phosphate buffer (pH 7.2) (membrane fraction). The resulting supernatant was used as the cytoplasmic/periplasmic (cp/pp) fraction.

Enzyme assays. (i) Plate assay. Esterase indicator plates (28) were prepared by addition of 15 ml of an emulsion of 50% (vol/vol) tributyrin and 5% (wt/vol) gum arabic to 500 ml of molten nutrient broth agar medium. Tributyrin was emulsified by sonication for 3 min at 75 W (duty cycle 100%) in a Branson 250 sonifier. Esterase and lipase activity is indicated by the formation of clear halos around the colonies.

(ii) Liquid assays. For esterase assay 1, 23.7 mg of *p*-nitrophenyl caproate (*p*NPC; Sigma) was dissolved in 10 ml of 2-propanol and added to 90 ml of Sørensen phosphate buffer (pH 8.0) supplemented with sodium deoxycholate (207 mg) and gum arabic (100 mg), yielding a final *p*NPC concentration of 1 mM. Culture supernatant (5 to 50 μl) was added to the substrate solution to give a final volume of 2.5 ml, the solution was incubated for 15 min at 30°C, and the OD_{410} was recorded with a Zeiss PMQ II spectrophotometer. The OD_{410}/OD_{580} ratio was used to estimate clone-specific extracellular enzyme activities during library screening. For esterase assay 2, 23.7 mg of *p*NPC was dissolved in ethanol (5 ml) and added to 95 ml of 100 mM potassium phosphate buffer (pH 7.0) containing 10 mM $MgSO_4$ to yield a final concentration of 1 mM *p*NPC. Samples (5 to 50 μl) were added to the substrate solution to give a final volume of 1 ml, and the ΔOD_{410}/min was recorded for 5 to 10 min at room temperature. The molar absorption coefficient of *p*NP at pH 7.0 was determined as 10,400 M^{-1}. One unit of enzyme activity is defined as the amount of enzyme forming 1 μmol of *p*NP per min. For the lipase assay 30 mg of *p*-nitrophenyl palmitate (*p*NPP; Sigma) was dissolved in 10 ml of 2-propanol at 60°C. The test was done as described above for esterase assay 1, except that the reaction was carried out at 37°C (63).

(iii) Glucose-6-phosphate dehydrogenase assay. Glucose-6-phosphate dehydrogenase was used as a cytoplasmic marker enzyme (10). A stock solution of NADP (45 mM) and a stock solution of glucose-6-phosphate (110 mM) were diluted 1:100 in a buffer containing 55 mM Tris-HCl (pH 7.5) and 11 mM $MgCl_2$. A 950-μl volume of this test solution was mixed with 50 μl of samples, and the decrease in optical density (ΔOD_{340}/min) was monitored spectrophotometrically at 30°C for 5 min.

Trypsin treatment of whole cells. *P. aeruginosa* and *E. coli* were grown overnight in LB medium. The cells were collected by centrifugation and resuspended to an OD_{580} of 5 in 100 mM Tris-HCl (pH 8.0). Trypsin (10 μl of a stock solution containing 2 mg of trypsin per ml in 100 mM Tris-HCl [pH 8.0]) was added to 100 μl of the cell suspension. After 1 h of incubation at 37°C, the protease was inhibited by the addition of 1 mM phenylmethylsulfonyl fluoride. Cells were collected by centrifugation and resuspended in 100 μl of 100 mM Tris-HCl (pH 8.0). Esterase activity was determined in supernatants and whole cells obtained after trypsin treatment. As a control, cells were treated in the same way except that no trypsin was added.

Selective labeling of plasmid-encoded proteins. The selective labeling of esterase encoded by pSKX$^{-/+}$ was performed as described previously (54). Bacteria precultured in LB/M9 medium were used to inoculated 5 ml of fresh medium to an OD_{580} of 0.1. At an OD_{580} of 0.6, the cells were harvested and added to 12 ml of M9 medium supplemented with 0.2% glucose and 0.2% methionine assay mix (Difco). *E. coli* BL21(DE3) harboring pUCPSK was used as a control. The expression of T7-RNA polymerase in *E. coli* BL21(DE3) (pLysS) was induced by the addition at $t = 0$ of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 30 min of incubation ($t = 30$), a sample was taken and *E. coli* RNA polymerase was inhibited by the addition of 200 μg of rifampin per ml. Additional samples were taken after 30 ($t = 60$) and 60 ($t = 90$) min of incubation with rifampin. These samples were labeled in vivo by supplementing 1 ml of culture with 1 μl of L-[^{35}S]methionine–L-[^{35}S]cysteine (10 μCi/ml; Amersham) and incubating the mixture for 10 min at 37°C. The cells were harvested, lysed, and denatured, and the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by autoradiographic detection on hyperfilm (Amersham) for 48 h.

SDS-PAGE. SDS-PAGE was performed as described by Laemmli (30) with a 5% stacking gel and a 15% separating gel.

DNA sequencing. DNA sequencing was kindly performed by Genencor International, Delft, The Netherlands, using the method described by Sanger et al. (43).

Software. The program BLAST X (2, 16) was used for protein homology searching, PSORT was used for prediction of protein localization (34), and DNAstar (Lasergene) was used for sequence alignments.

Nucleotide sequence accession number. The DNA sequence of *estA* from *P. aeruginosa* has been deposited in GenBank (accession no. AF005091).

RESULTS AND DISCUSSION

Screening of a genomic library. A genomic library containing *P. aeruginosa* DNA cloned in pLAFR3 was transferred from *E. coli* S17-1 into the lipase-negative mutant *P. aeruginosa* PABS1 by conjugation, and bacteria were plated on esterase indicator plates containing tributyrin. Although this triglyceride is a classical lipase substrate, it is partially soluble in water and is therefore also hydrolyzed by esterases (7). Colonies with extracellular esterase or lipase activity formed clear halos on these plates. As shown in Fig. 1A, lipase production in the wild-type strain *P. aeruginosa* PAO1 caused the formation of a large halo, which appeared after overnight incubation, whereas the halo formed by the lipase-negative mutant PABS1 was much smaller and appeared only after incubation of the plates for at least 48 h. Our screening was based on the assumption that such a halo would appear earlier and with increased diameter if several copies of the esterase gene cloned in pLAFR3 were expressed in the lipase-negative mutant. After a library consisting of 15,000 clones was screened, 11 positive clones with halos of intermediate size, i.e., smaller than that formed by the wild type but larger than that of the lipase-negative mutant, were identified. Several clones identified on esterase indicator plates were further assayed for lipase and esterase activity in the culture supernatant (Fig. 1B). The wild-type strain, PAO1, showed high lipase activity and significantly lower esterase activity. However, its esterase activity was still higher than that of the lipase-negative mutant, PABS1, presumably because of the ability of lipase to cleave the esterase substrate. Introduction of pLAFR3-21.P into *P. aeruginosa* PABS1 yielded a 10-fold increase in esterase activity (Fig. 1B). The slightly increased lipase activity was presumably caused by the ability of esterase to hydrolyze the lipase substrate *p*-nitrophenylpalmitate.

Identification of the esterase. The cosmid pLAFR3-21.P was digested with various restriction endonucleases, and fragments were subcloned into plasmid pUCPSK, transferred into the lipase-negative mutant, PABS1, and assayed for esterase activity. After several subcloning steps, one clone containing plasmid pUCPSK carrying a 3.3-kb *Xho*I fragment was isolated. This fragment was cloned in both orientations, and the corresponding plasmids were named pSKX+ and pSKX−, respectively. *P. aeruginosa* PABS1(pSKX+) showed high esterase activity (data not shown), whereas no increase in esterase activity was observed for *P. aeruginosa* PABS1(pSKX−). The DNA fragment cloned in plasmid pUCPSK in the positive orientation is expressed from the *lac* promoter, which is constitutively expressed in *P. aeruginosa*, thereby explaining the observed esterase activity, whereas in the opposite (negative) orientation it is under control of the T7 promoter. To identify a putative protein encoded by this fragment, we used an *E. coli* T7-RNA polymerase expression system. Samples were taken at several time intervals, and each of them was immediately labeled with L-[^{35}S]methionine–L-[^{35}S]cysteine in vivo. Expression from pSKX− yielded a single 66-kDa protein band visible upon autoradiography, which appeared when cellular protein synthesis was inhibited by addition of rifampin (Fig. 2). Since no protein bands were detected in cells harboring either the control plasmid pUCPSK or pSKX+, we concluded that the 3.3-kb *Xho*I fragment of *P. aeruginosa* DNA cloned in pSKX− contained a single open reading frame (ORF) expressed from P_{T7} which encoded a protein with M_r = 66,000.

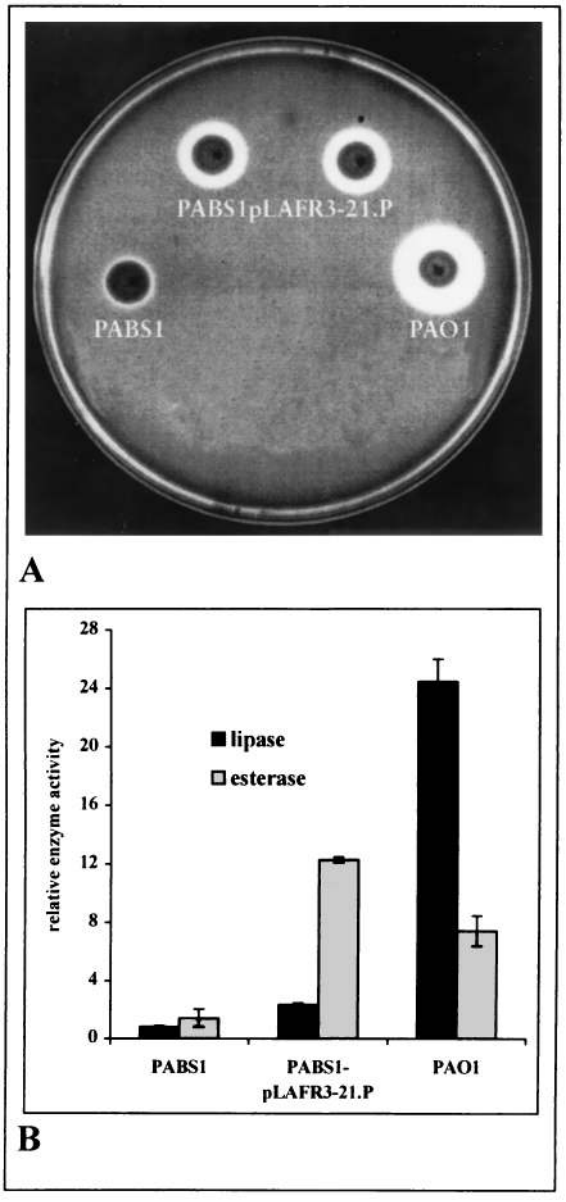

FIG. 1. Lipolytic activity of *P. aeruginosa* strains, wild-type PAO1, lipase-negative mutant PABS1, and PABS1pLAFR3-21.P containing a cosmid with a 22.1-kb insert of chromosomal DNA from *P. aeruginosa* PAO1. (A) Halo formation of bacterial colonies on esterase indicator plates after incubation for 3 days at 30°C. (B) Lipolytic activities of culture supernatants in liquid assays, assayed with *p*-nitrophenylpalmitate (C_{16}) for lipase activity and *p*-nitrophenylcaproate (C_6) for esterase activity. Relative enzyme activities were determined as the ratio of OD_{410} (enzyme activity) to OD_{580} (cell density) per milliliter of culture supernatant.

Nucleotide sequence analysis. Determination of the nucleotide sequence of this DNA fragment (Fig. 3) revealed an ORF of 1,941 bp, which was designated *estA*. A putative Shine-Dalgarno sequence was located 7 bp upstream of the ATG start codon, and a consensus sequence typical for an RpoN-dependent promoter [TGGCACN$_5$TTGC(a/t)] (33) was identified 135 bp upstream of the translational start codon. The G+C content was 66.9% and the frequency of C or G at the

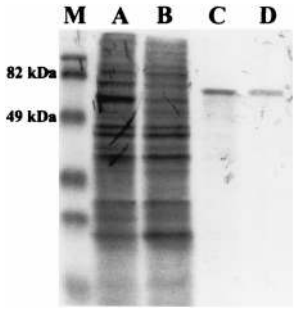

FIG. 2. Autoradiography of L-[^{35}S]methionine–L-[^{35}S]cysteine-labeled proteins from cell lysates of *E. coli* BL21(DE3)(pLysS) containing pSKX− separated by SDS-PAGE. Samples were labeled prior to induction of T7 RNA polymerase expression (t = 0; lane A), 30 min after induction but prior to inhibition of *E. coli* RNA polymerase with rifampin (t = 30; lane B), and 30 min (t = 60; lane C) and 60 min (t = 90; lane D) after addition of rifampin. Lane M contains prestained molecular mass markers (Bio-Rad).

third codon position was 89.2%, indicating a typical *P. aeruginosa* codon usage (62). The esterase encoded by *estA* is synthesized as a 646-amino-acid precursor with a calculated M_r of 69,526, including a predicted 24-amino-acid signal sequence (35). The mature esterase has a calculated molecular weight of 67,000, which is consistent with the M_r determined by SDS-PAGE and autoradiography. A BLAST X (2, 16) analysis of *estA* revealed homologies to an ORF located in the *trpE-trpG* region of *P. putida* (11), to *apeE* encoding an outer membrane esterase from *Salmonella typhimurium* (9), to *lip-1* encoding a lipase from *Xenorhabdus* (*Photorhabdus*) *luminescens* (59), and to the GCAT gene encoding a acyltransferase of *Aeromonas hydrophila* (32). These proteins and the esterase of *P. aeruginosa* belong to a novel family of lipolytic enzymes identified on the basis of sequence homology (55). Many of the prokaryotic proteins of this new family exhibited lipolytic activity; eukaryotic members were found in higher plants (6), and their physiological functions are as yet unknown. Five conserved blocks of high amino acid homology were identified (Fig. 4). The enzymes belonging to this novel family differ from other lipases in the location and structure of the active-site consensus motif G-X-S-X-G (22). In this family, this motif is located close to the N terminus and consists of G-D-S-X-S, with the terminal glycine of the characteristic lipase consensus motif replaced in most cases by serine. The active site of these enzymes consists of a catalytic triad formed by the amino acids serine, histidine, and aspartate. For *A. hydrophilia* lipase/acyltransferase, serine 16, histidine 291, and aspartate 116, located in blocks I, V, and

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1    GAAGAAGATCGGCCTGTGGGCGGTACTTCTGCTGGGGGTCGCTCTGCTGGTAATAATGGCAATGAGCCTGCTGCGTTCCTCGCCGAACAAGCCATGAGCC
101  GGTTCCGCGCTATGCTGTTCGGCTGAGGAGGCTTTACGACGGGCCCCGGGGCGCATGCCGACGACGCGGCGGCCCGACAATAAAAACAAATCATGGAGTA
201  AGAGAATGATCAGAATGGCGCTCAAGCCACTGGTAGCGGCCTGCCTGCTGGCTTCGCTGTCCACCGCCCCGCAGGCTGCTCCTTCGCCCTATTCGACGCT
                  M  I  R  M  A  L  K  P  L  V  A  A  C  L  L  A  S  L  S  T  A  P  Q  A  A  P  S  P  Y  S  T  L
301  GGTGGTGTTCGGCGACAGCCTCAGCGATGCCGGGCAGTTCCCCGATCCTGCCGGCCCCGCCGGAAGCACCTCGCGTTTCACCAACCGGGTCGGCCCGACC
       V  V  F  G  D  S  L  S  D  A  G  Q  F  P  D  P  A  G  P  A  G  S  T  S  R  F  T  N  R  V  G  P  T
401  TACCAGAACGGCAGCGGCGAGATCTTCGGACCGACCGCGCCCATGCTGCTCGGCAATCAGCTCGGCATCGCCCCGGGTGACCTGGCTGCCTCGACCTCGC
       Y  Q  N  G  S  G  E  I  F  G  P  T  A  P  M  L  L  G  N  Q  L  G  I  A  P  G  D  L  A  A  S  T  S
501  CGGTCAACGCCCAGCAGGGCATCGCCGACGGCAACAACTGGGCGGTGGGCGGCTACCGGACCGATCAGATCTACGACTCGATCACCGCGGCCAACGGCTC
       P  V  N  A  Q  Q  G  I  A  D  G  N  N  W  A  V  G  G  Y  R  T  D  Q  I  Y  D  S  I  T  A  A  N  G  S
601  GCTGATCGAGCGCGACAATACCCTCCTGCGCAGCCGCGATGGCTACCTGGTGGACCGTGCCCGCCAGGGCCTGGGTGCCGACCCGAACGCGTTGTACTAC
       L  I  E  R  D  N  T  L  L  R  S  R  D  G  Y  L  V  D  R  A  R  Q  G  L  G  A  D  P  N  A  L  Y  Y
701  ATCACCGGCGGCGGCAACGACTTTCTCCAGGGGCGCATCCTCAACGACGTCCAGGCCCAACAGGCCGCCGGTCGCCTGGTGGATAGCGTGCAGGCCCTGC
       I  T  G  G  G  N  D  F  L  Q  G  R  I  L  N  D  V  Q  A  Q  Q  A  A  G  R  L  V  D  S  V  Q  A  L
801  AGCAGGCCGGCGCGCGCTACATCGTGGTCTGGCTGTTGCCCGACCTGGGCCTGACCCCGGCTACCTTCGGTGGTCCCTTGCAGCCTTTCGCCAGCCAACT
       Q  Q  A  G  A  R  Y  I  V  V  W  L  L  P  D  L  G  L  T  P  A  T  F  G  G  P  L  Q  P  F  A  S  Q  L
901  CAGCGGCACGTTCAACGCCGAGCTGACCGCCCAGTTGAGCCAGGCCGGCGCCAACGTCATTCCGTTGAACATCCCGCTGCTGCTCAAGGAAGGCATGGCC
       S  G  T  F  N  A  E  L  T  A  Q  L  S  Q  A  G  A  N  V  I  P  L  N  I  P  L  L  L  K  E  G  M  A
1001 AACCCGGCTTCCTTCGGCCTGGCCGCCGACCAGAACCTGATCGGCACCTGTTTCAGCGGCAACGGCTGCACCATGAACCCGACCTACGGGATCAACGGCA
       N  P  A  S  F  G  L  A  A  D  Q  N  L  I  G  T  C  F  S  G  N  G  C  T  M  N  P  T  Y  G  I  N  G
1101 GCACGCCCGACCCGAGCAAATTGCTGTTCAACGACAGCGTGCACCCGACCATCACCGGCCAGCGCCTGATCGCCGACTACACCTATTCGCTGCTGTCGGC
       S  T  P  D  P  S  K  L  L  F  N  D  S  V  H  P  T  I  T  G  Q  R  L  I  A  D  Y  T  Y  S  L  L  S  A
1201 GCCCTGGGAGCTGACCCTGCTGCCGGAAATGGCCCACGGCACCCTGCGTGCCTACCAGGACGAACTGCGCAGCCAGTGGCAGGCGGACTGGGAGAACTGG
       P  W  E  L  T  L  L  P  E  M  A  H  G  T  L  R  A  Y  Q  D  E  L  R  S  Q  W  Q  A  D  W  E  N  W
1301 CAGAACGTCGGCCAGTGGCGCGGCTTCGTCGGCGGCGGTGGCCAGCGCCTGGACTTCGACTCCCAGGACAGCGCCGCCAGCGGCGACGGCAACGGCTACA
       Q  N  V  G  Q  W  R  G  F  V  G  G  G  G  Q  R  L  D  F  D  S  Q  D  S  A  A  S  G  D  G  N  G  Y
1401 ACCTGACCCTTGGTGGCAGCTACCGCATCGACGAGGCCTGGCGCGCCGGGGTCGCCGCCGGTTTCTACCGGCAGAAGCTGGAAGCCGGCGCCAAGGATTC
       N  L  T  L  G  G  S  Y  R  I  D  E  A  W  R  A  G  V  A  A  G  F  Y  R  Q  K  L  E  A  G  A  K  D  S
1501 CGACTACCGGATGAACAGCTACATGGCCAGCGCCTTCGTGCAGTACCAGGAAAACCGCTGGTGGGCCGACGCGGCGTTGACCGGCGGCTACCTCGACTAC
       D  Y  R  M  N  S  Y  M  A  S  A  F  V  Q  Y  Q  E  N  R  W  W  A  D  A  A  L  T  G  G  Y  L  D  Y
1601 GACGACCTGAAGCGCAAGTTCGCCCTGGGCGGCGGCGAGCGCAGCGAGAAAGGCGACACCAACGGCCACCTGTGGGCGTTCAGCGCGCGCCTGGGCTACG
       D  D  L  K  R  K  F  A  L  G  G  G  E  R  S  E  K  G  D  T  N  G  H  L  W  A  F  S  A  R  L  G  Y
1701 ACATCGCCCAGCAGGCCGACAGTCCCTGGCACCTGTCGCCGTTCGTCAGCGCCGACTATGCACGGGTCGAGGTCGACGGCTATTCCGAGAAGGGCGCCAG
       D  I  A  Q  Q  A  D  S  P  W  H  L  S  P  F  V  S  A  D  Y  A  R  V  E  V  D  G  Y  S  E  K  G  A  S
1801 CGCCACCGCGCTCGACTACGACGACCAGAAGCGCAGCTCGAAGCGCCTGGGCGCCGGCCTGCAAGGCAAGTACGCGTTCGGCAGCGATACCCAGCTGTTC
       A  T  A  L  D  Y  D  D  Q  K  R  S  S  K  R  L  G  A  G  L  Q  G  K  Y  A  F  G  S  D  T  Q  L  F
1901 GCCGAGTACGCCCACGAACGTGAGTACGAGGACGACACCCAGGACCTGACCATGTCCCTCAACAGCCTGCCGGGCAATCGCTTCACCCTCGAAGGCTACA
       A  E  Y  A  H  E  R  E  Y  E  D  D  T  Q  D  L  T  M  S  L  N  S  L  P  G  N  R  F  T  L  E  G  Y
2001 CCCCGCAGGACCATCTCAACCGCGTCTCACTCGGCTTCAGCCAGAAGCTGGCGCCGGAGCTGTCGCTGCGCGGCGGCTACAACTGGCGCAAGGGCGAGGA
       T  P  Q  D  H  L  N  R  V  S  L  G  F  S  Q  K  L  A  P  E  L  S  L  R  G  G  Y  N  W  R  K  G  E  D
2101 CGATACCCAGCAGAGCGTCAGCCTGGCGCTGAGCCTGGACTTCTGAAACGGCGGCCGGCGCCCGGTCGGCGCC
       D  T  Q  Q  S  V  S  L  A  L  S  L  D  F  .
```

FIG. 3. Nucleotide sequence and derived amino acid sequence of the esterase gene *estA*. The putative Shine-Dalgarno sequence is underlined, the consensus sequence for the RpoN (σ^{54})-dependent promoter is marked by dashed lines, and the putative signal sequence is indicated by an arrow.

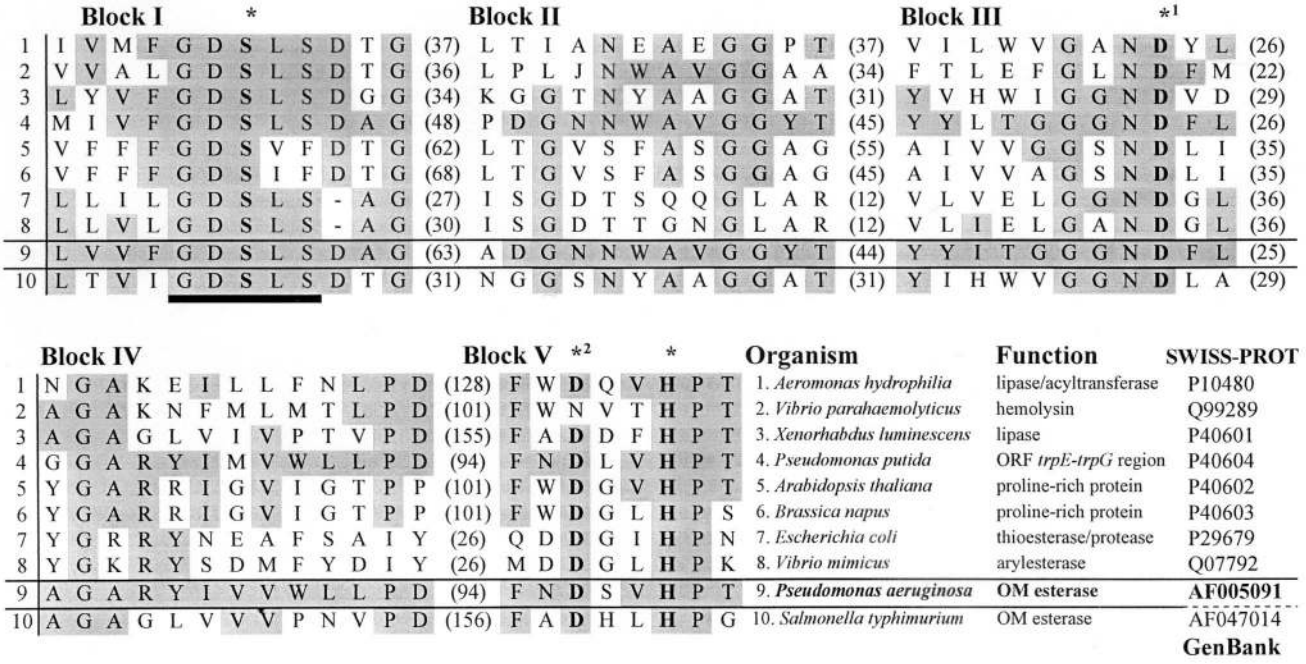

FIG. 4. Sequence comparison between *P. aeruginosa* esterase (EstA) and members of a novel family of lipolytic enzymes (55). Identical amino acids are shaded in grey; numbers in parentheses refer to the number of amino acid residues between the conserved blocks. The putative catalytic triad residues (*) are printed in bold, and the G-D-S-L-S consensus motif is underlined.

III, respectively (Fig. 4), were shown by site-directed mutagenesis to form the catalytic triad (8). Aspartate 116 could not unequivocally be assigned to the active site, and a second aspartate residue at position 288 was identified as another likely candidate (8). Recently, the three-dimensional structure of an esterase from *Streptomyces scabies* was determined by X-ray crystallography (61). This enzyme is unique among all known lipolytic enzymes in that instead of a triad, it contains an active-site dyad consisting of a serine and a histidine residue. The correct orientation of the histidine imidazole ring is

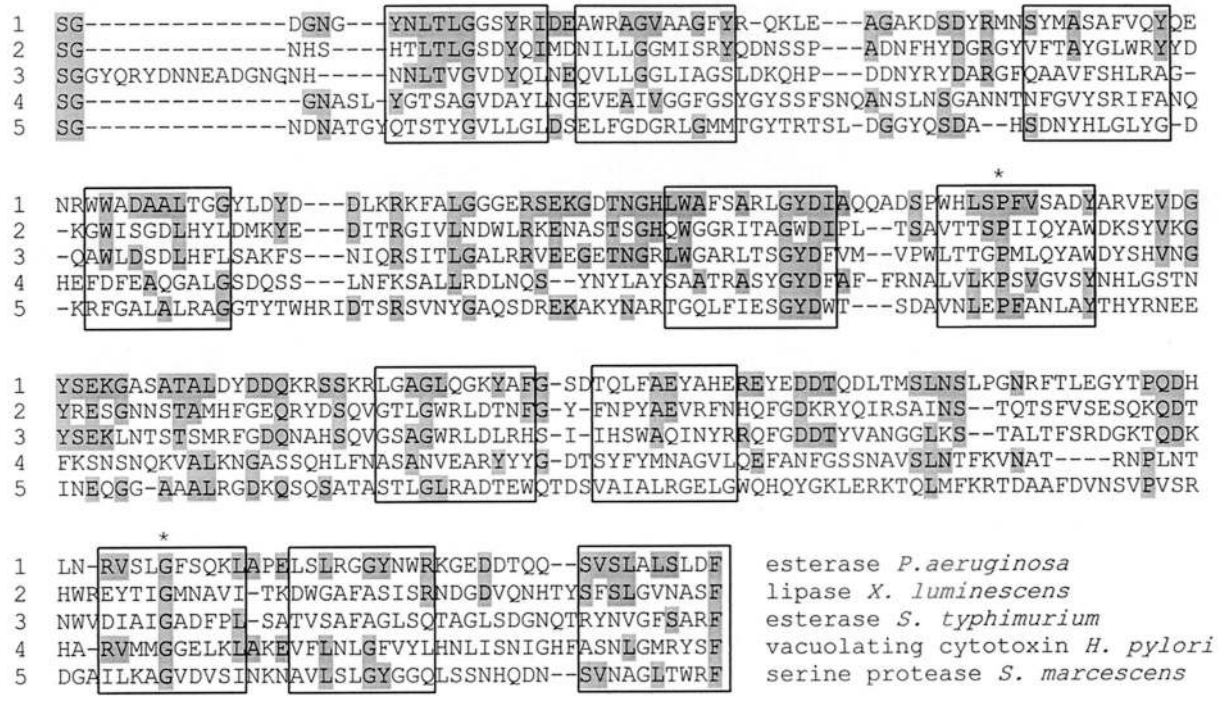

FIG. 5. Multiple alignment of C-terminal domains. EstA, outer membrane proteins, and secreted proteins belonging to the autotransporter family (31) were compared. Boxes indicate putative amphiphathic β-barrels, and the two fully conserved amino acids from the ATF are marked with an asterisk.

TABLE 2. Extracellular enzyme activities of a lipase-negative *P. aeruginosa* strain and mutants defective in the general secretory pathway

Mutant	Genotype	Gene expressed from plasmid	Relative enzyme activity (%)[a]	
			Esterase	Lipase
PABS1	*lipA*	*estA*	100	17
2B18	*xcpA*	*estA*	97	0
PUS13	*xcpQ*	*estA*	98	0
PABS1	*lipA*	*lipA*	15	100
2B18	*xcpA*	*lipA*	0	0
PUS13	*xcpQ*	*lipA*	0.1	0

[a] Extracellular enzyme activities of *P. aeruginosa* PABS1 harboring either the cloned esterase gene on pSKX+ or the lipase gene on pLip1 were arbitrarily set at 100%. Relative enzyme activities were determined as the ratio of OD_{410} (enzyme activity) to OD_{580} (cell density) per milliliter of culture supernatant with the substrates *p*-NPC for esterase and *p*-NPP for lipase.

normally ensured by hydrogen bonding to the carboxyl group of a third residue (aspartate or glutamate), which is replaced here by the backbone carbonyl of a tryptophan. The three-dimensional structure of an esterase isolated from bovine brain has a similar active-site architecture. In this case, however, the tryptophan is indeed replaced by aspartate (18). A comparison of the amino acid sequences of *S. scabies* and bovine brain esterases with the sequences of *A. hydrophila* lipase/acyltransferase and of *P. aeruginosa* EstA (3) indicated that in these enzymes, aspartate residues at positions 288 and 286, respectively, belong to the catalytic triad, as indicated in Fig. 4. Therefore, we predict that the catalytic triad of the *P. aeruginosa* esterase is formed by serine 14, histidine 289, and aspartate 286.

Secretion. Esterase activity could be detected in the bacterial culture supernatant (Fig. 1B). Furthermore, a putative signal sequence precedes the mature esterase protein, leading us to assume that this enzyme could be secreted by the general secretory pathway (GSP) (12). This pathway consists of two steps: the secreted protein is translocated through the inner membrane via the Sec-dependent mechanism and is subsequently translocated through the outer membrane by the Xcp machinery (12). The lipase-negative mutant *P. aeruginosa* PABS1 and two different *xcp* mutants were transformed with plasmids encoding either lipase (pLip1) or esterase (pSKX+), and the extracellular enzyme activities were determined. In mutant 2B18, the *xcpA* gene, encoding the prepilin-peptidase, which is required for the processing of several Xcp components, is disrupted (36, 49), and in mutant PUS13, the outer membrane secretin XcpQ is absent. Since the lipase is known to be secreted via the GSP (24), it served as a control. As shown in Table 2, *xcp* mutants did not show extracellular lipase activity. However, the extracellular esterase activity remained unaffected, demonstrating that the esterase is not secreted via the GSP.

Cellular localization. EstA was predicted to be anchored to the outer membrane by the computer program PSORT (34). Additional observations suggested an outer membrane location of the 28-kDa C-terminal fragment of EstA (amino acids 374 to 622): (i) it contains many putative amphipathic β-strands, and (ii) as in the vast majority of bacterial outer membrane proteins (50), the C-terminal amino acid residue is a phenylalanine. These observations led us to compare the C-terminal fragment of EstA with other outer membrane proteins (Fig. 5), revealing a significant similarity to a family of proteins which is secreted by gram-negative bacteria and has been designated the autotransporter family (31). These proteins, which are believed to be virulence factors, are secreted by the so-called type IV secretion mechanism (13), which has so far not been found to operate in *P. aeruginosa* (17). Autotransporter proteins are translocated through the inner membrane via a Sec-dependent mechanism and cross the periplasm, and their β-domain inserts into the outer membrane. The catalytically active α-domain (i) remains attached to the outer membrane, (ii) is autoproteolytically cleaved off, or (iii) is cleaved off by another protease (17). Significant amino acid homologies to recognized members of the autotransporter family, such as Ssp (22.6%), a serine protease from *Serratia marcescens* (64), and VacA (23.3%), a vacuolating cytotoxin from *Helicobacter pylori* (44), were detected. However, the

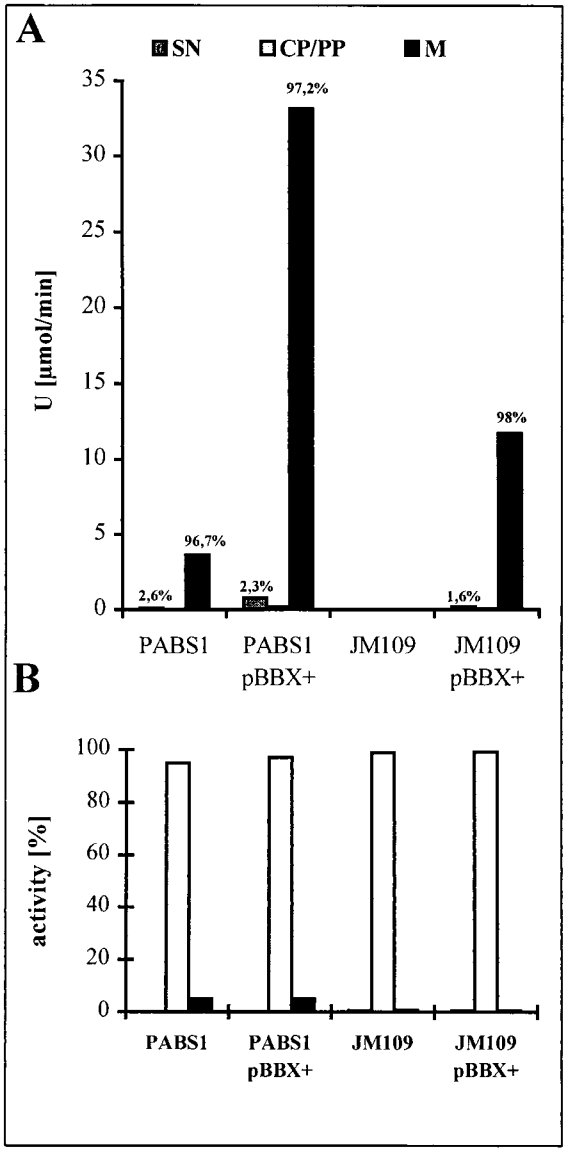

FIG. 6. Distribution of esterase (A) and glucose-6-phosphate dehydrogenase (B) activity in cellular compartments of *P. aeruginosa* PABS1 and *E. coli* JM109 containing *estA* on pBBX+. The percentages of total enzyme activities present in culture supernatants (SN), cytoplasmic-periplasmic fractions (CP/PP), and membranes (M) are given.

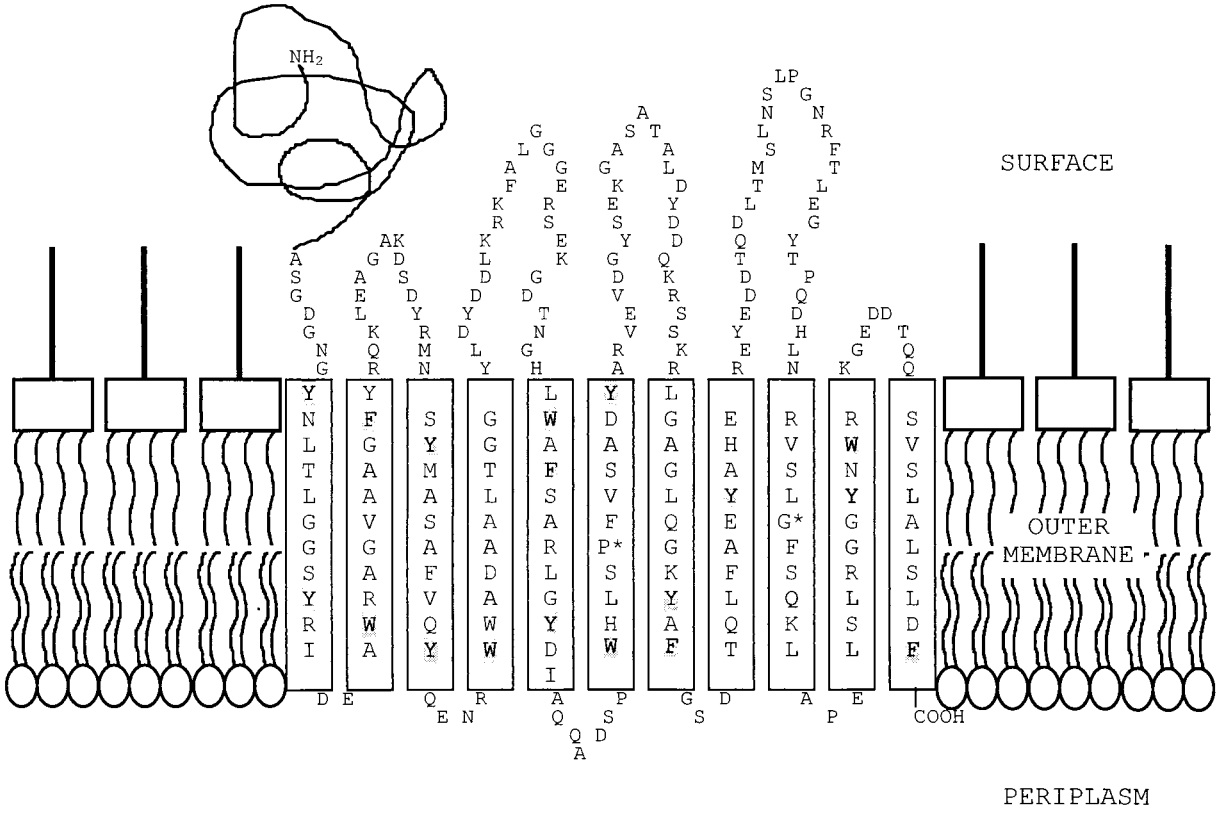

FIG. 7. One possible model of the C-terminal domain of *P. aeruginosa* esterase (EstA) located in the outer membrane. The C-terminal domain (~G 374 to F 622) is predicted to consist of 11 amphipathic β-strands with 10 to 12 amino acid residues per strand, sufficient to traverse the hydrophobic core of the membrane. The two amino acids fully conserved in proteins belonging to the autotransporter family (31) are marked with asterisks; amino acids printed in bold indicate the hydrophobic side of the β-strands; and amino acids shaded in grey represent two girdles of aromatic residues, which seem to be present in outer membrane proteins of known structure.

highest homology scores were found to an outer membrane-located esterase from *S. typhimurium* (24.6%) (9) and to a secreted lipase from *X. luminescens* (29.3%) (59). Interestingly, this lipase inserts into the outer membrane upon expression in *E. coli* (59). So far, these lipolytic enzymes have not been found to possess a C-terminal autotransporter domain. Autotransporters share a number of characteristic features, which also exist in the *P. aeruginosa* esterase. (i) They possess an N-terminal leader peptide. (ii) The mature protein consists of a surface-exposed N-terminal passenger domain (or α-domain), which harbors the catalytic site, and a C-terminal β-domain located in the outer membrane (17). The α-domain usually contains the N-terminally located active-site motifs followed by a stretch of amino acids with few cysteine residues (Cys258 and Cys264 in EstA). The C-terminal β-domains of autotransporters are predicted to contain 14 amphipathic β-strands, which may form a β-barrel pore, allowing the translocation of the α-domain to the bacterial cell surface (31). (iii) The terminal amino acid residue is always a phenylalanine or tryptophan, which is preceded by four alternating charged/polar and hydrophobic/aromatic residues (31, 50). In EstA the terminal amino acid residue is F622 and is preceded by D-L-S-L-A-L-S-V. (iv) Proline 171 and glycine 275 have been identified as two fully conserved residues by the aligning autotransporter domains of several proteins (31). These residues are also present in EstA (Fig. 5).

Based on these predictions, we investigated experimentally the localization of EstA in the lipase-negative mutant *P. aeruginosa* PABS1 (wild type for esterase) and the esterase-overexpressing strain *P. aeruginosa* PABS1 containing *estA* on pBBX+. Cellular compartments were fractionated into culture supernatant, cytoplasmic-periplasmic fraction, and a crude membrane fraction. The absolute esterase activity was about 10-fold higher in the overexpressing strain, but in both strains more than 95% of the esterase activity was detected in the crude membrane fraction (Fig. 6A). Only 2 to 3% of the enzyme activity was released into the culture supernatant, which did not contain glucose-6-phosphate dehydrogenase activity (Fig. 6B), indicating that no significant cell lysis had occurred. These results showed that EstA is tightly membrane bound in *P. aeruginosa*. A comparable result was obtained when *estA* was expressed in the heterologous host *E. coli*, although the absolute amounts of esterase activity were smaller. The small amount of extracellular esterase may have two possible explanations: (i) the enzyme was cleaved by a protease and remained bound to the outer membrane, as found for the Ag43 protein from *E. coli* (39), with only a small amount released into the extracellular medium; or (ii) the enzyme remained bound to the outer membrane, as shown for Hsr from *Helicobacter mustelae* (38). A small part of the esterase might have been released either in a free form or in the form of membrane vesicles which contain in *P. aeruginosa* periplasmic, membrane-bound, and extracellular proteins (4).

Inspection of the C-terminal domain of EstA revealed several segments that could form amphipathic β-strands. Assuming that the β-domains of all autotransporters have similar

tertiary structures, we expect that the amphipathic character should be conserved in all β-strands that are actually part of the β-barrel. When the new members of the autotransporter family identified in this study are aligned with the recognized members of the family (examples are given in Fig. 5), 11 segments that could form amphipathic β-strands in all sequences could be distinguished. These segments are almost devoid of β-turn predictions, when the criteria described by Paul and Rosenbusch (40) are applied, whereas turn predictions are generally found in the intervening segments (data not shown). Since a closed β-barrel should consist of an even number of β-strands, the β-barrel may consist of as few as 10 β-strands, with the first segment reaching through the interior of the barrel, thereby exposing the α-domain to the cell surface. Alternatively, the barrel formed may not be closed, creating an instability that may result in the destruction of the β-domain after the translocation of the α-domain. In this respect, it should be noted that the fate of the β-domain after having performed its job has hardly been investigated so far. A 10- or 11-stranded β-barrel could enclose only a small pore, which indicates that the passenger domain should be transported in a delineated fashion. In agreement, the periplasmic folding of an artificial passenger domain has been demonstrated to prevent outer membrane translocation (26). However, it should be noted that the β-domain does not necessarily have to form a β-barrel with an enclosed pore to exert its function. The molecular mechanism of transport may be entirely different. For example, it has been reported that a lipid-modified domain of OmpA, encompassing only five β-strands, can transport a periplasmic passenger protein to the cell surface (14). Clearly, these five β-strands cannot form a pore through which the passenger is transported. A tentative topology model of the β-domain of EstA is depicted in Fig. 7. As in the porins, the β-strands are connected by short periplasmic turns and longer extracellular loops. Furthermore, two girdles of aromatic residues that may surround the putative β-barrel at the height of the polar head groups of the lipids in the membrane can readily be discerned. Further experiments are required to investigate the structure and function of the autotransporter domain of *P. aeruginosa* EstA as well as of other members of the family.

Our model predicts that the catalytically active N-terminal domain of EstA is exposed to the surface and should at least partly be accessible to proteolytic cleavage. Therefore, whole cells of *P. aeruginosa* and *E. coli* expressing *estA* were treated with trypsin and the residual cell-bound esterase activity was determined. As shown in Fig. 8 a significant amount of esterase activity was removed (20% for *P. aeruginosa* and 10% for *E. coli*). Increasing the amount of trypsin did not result in an increased amount of esterase removed from cells. Increasing the concentration of $MgCl_2$ during trypsin treatment led to a decrease in the amount of esterase activity removed, whereas increasing the concentration of EDTA led to a increase in the amount of esterase activity removed (data not shown). These results indicate the following. (i) The esterase expressed in *P. aeruginosa* and in *E. coli* is partly accessible to trypsin treatment of whole cells. Since the localization and the protease accessibility in *E. coli* and in *P. aeruginosa* are very similar, the information allowing the enzyme to reach the cell surface must be independent of specific factors present only in the homologous host and should therefore reside in EstA itself. (ii) Removal of a significant part of the esterase activity from whole cells of *P. aeruginosa* and *E. coli* by trypsin treatment strongly suggests that EstA is attached to the bacterial outer membrane, as predicted by our model (Fig. 7). (iii) The trypsin-resistant portion of the esterase may be shielded by association with lipopolysaccharide and/or exopolysaccharide as described for outer membrane proteins (48) and also for extracellular lipase from *P. aeruginosa* (53). Further experiments are required to decide whether the accessibility of esterase to trypsin is different in appropriate mutants altered in lipopolysaccharide or exopolysaccharide (e.g., alginate) composition.

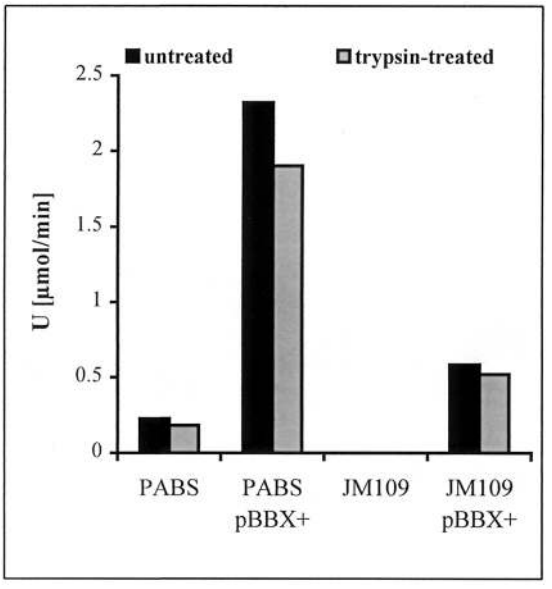

FIG. 8. Effect of trypsin treatment on esterase activities of whole cells of *P. aeruginosa* and *E. coli* containing *estA* on pBBX+.

In summary, a novel esterase was identified which is located in the outer membrane of *P. aeruginosa*. This enzyme presumably belongs to a family of putative virulence factors which are self-secreted via a C-terminally located autotransporter domain. Determination of esterase activity in a cell-free culture supernatant led us to assume that the catalytically active N-terminal esterase domain may be released into the external medium by a so far unknown mechanism. At present, we are trying to identify the enzyme responsible for proteolytic cleavage of surface-exposed EstA and are investigating the physiological significance of this novel lipolytic enzyme.

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