JOURNAL OF BACTERIOLOGY, July 1998, p. 3517–3521 Vol. 180, No. 14
0021-9193/98/$04.00+0

The *apeE* Gene of *Salmonella typhimurium* Encodes an Outer Membrane Esterase Not Present in *Escherichia coli*

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Received 23 February 1998/Accepted 3 May 1998

***Salmonella typhimurium apeR* mutations lead to overproduction of an outer membrane-associated *N*-acetyl phenylalanine β-naphthyl ester-cleaving esterase that is encoded by the *apeE* gene (P. Collin-Osdoby and C. G. Miller, Mol. Gen. Genet. 243:674–680, 1994). This paper reports the cloning and nucleotide sequencing of the *S. typhimurium apeE* gene as well as some properties of the esterase that it encodes. The predicted product of *apeE* is a 69.9-kDa protein which is processed to a 67-kDa species by removal of a signal peptide. The predicted amino acid sequence of ApeE indicates that it is a member of the GDSL family of serine esterases/lipases. It is most similar to a lipase excreted by the entomopathogenic bacterium *Photorhabdus luminescens*. The *Salmonella* esterase catalyzes the hydrolysis of a variety of fatty acid naphthyl esters and of C_6 to C_{16} fatty acid *p*-nitrophenyl esters but will not hydrolyze peptide bonds. A rapid diagnostic test reported to be useful in distinguishing *Salmonella* spp. from related organisms makes use of the ability of *Salmonella* to hydrolyze the chromogenic ester substrate methyl umbelliferyl caprylate. We report that the *apeE* gene product is the enzyme in *Salmonella* uniquely responsible for the hydrolysis of this substrate. Southern blot analysis indicates that *Escherichia coli* K-12 does not contain a close analog of *apeE*, and it appears that the *apeE* gene is contained in a region of DNA present in *Salmonella* but not in *E. coli*.**

Mutations at the *apeA* locus of *Salmonella typhimurium* lead to loss of a periplasmic enzyme originally identified by its ability to hydrolyze the chromogenic substrate *N*-acetyl phenylalanine β-naphthyl ester (NAPNE) (17). Mutants with a reduced capacity for NAPNE hydrolysis are easily isolated by using this substrate to detect activity in situ in bacterial colonies growing on an agar surface. NAPNE is a good substrate for chymotrypsin, and ApeA was originally thought to be a protease, protease I (19, 20). Recent work indicates that the *Escherichia coli apeA* product is a thioesterase (4), and the gene is now designated *tesA*.

We have previously described the isolation of pseudorevertants of *S. typhimurium apeA* mutations that lead to restoration of the ability to hydrolyze NAPNE (6). These mutants overproduce a membrane-associated enzyme which is distinct from ApeA and all other known NAPNE hydrolases (12). The mutations affect a locus, *apeR*, that appears to encode a negative regulator of the transcription of the membrane hydrolase which is thought to be the product of the *apeE* gene (6). To further characterize this membrane hydrolase, we report the cloning and nucleotide sequence of the *apeE* gene and further characterization of the enzymatic activity of its product, ApeE.

MATERIALS AND METHODS

Bacterial strains. Table 1 lists the *S. typhimurium* LT2 strains used in this project. Other strains used were *Photorhabdus luminescens* K122 (obtained from Barbara Dowds, St. Patrick's College, County Kildare, Ireland), *Pseudomonas aeruginosa* K (obtained from David Nunn, University of Illinois), and derivatives of *E. coli* K-12.

Media and growth conditions. *S. typhimurium* and *E. coli* strains were routinely grown at 37°C in Luria broth medium (Gibco BRL) aerated by shaking. *P. luminescens* was grown under the same conditions but at 28°C. Antibiotics were added as indicated in the following concentrations: ampicillin, 50 mg/ml; chloramphenicol, 15 mg/ml; and tetracycline, 25 mg/ml (18). Minimal (E min) soft agar overlays were prepared from E medium (30) with 0.8% Difco agar.

Isolation of plasmids carrying *apeE*. Plasmids containing 8- to 15-kb fragments generated by *Sau*3A partial digestion of DNA from *S. typhimurium* TN1379 and inserted into the *Bam*HI site of pBR328 were transformed into strain TN2540. To screen this library, a P22HT 12/4 int-3 lysate was made on the library and used to transduce TN445, an *apeA apeE*$^+$ *apeR*$^+$ strain, with selection for chloramphenicol resistance. These transductants were screened for NAPNE-hydrolyzing activity by overlaying the transduction plate with 2.5 ml of E min soft agar; after solidification, 10 ml of a mixture containing 0.1 M phosphate buffer (pH 6.8), 10 mg of Fast Garnet GBC (Sigma), and 2 mg of NAPNE in *N*,*N*-dimethylformamide (final concentration is 10%) was poured over the plate, and the plate was incubated at room temperature for about 1 min. The plates were then washed with sterile saline, and any NAPNE-staining clones were picked and restreaked.

Subcloning. Subclones of pCM342 were obtained by partial digestion of the plasmid with *Sau*3A, ligation into the *Bam*HI site of pBR328, and electroporation into *E. coli* DH5α. The transformants were stained for activity as described above and restreaked. Although DH5α is *apeA*$^+$, it is easy to distinguish the dark red color of a colony containing an *apeE* plasmid from the pink color of the parental colony. The presence of an *apeE* plasmid in the red-staining strains was confirmed by staining sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) gels for NAPNE activity after renaturation (see below).

DNA sequencing. DNA sequencing was carried out with a Sequenase version 2.0 DNA sequencing kit (U.S. Biochemicals) according to manufacturer's instructions. Compressed areas were resolved by automated cycle sequencing by the University of Illinois Biotechnology Center. The DNA was completely sequenced in both strands. Sequence alignment and analysis were carried out with the Wisconsin sequence analysis package (Genetics Computer Group [GCG]) and the Lasergene software DNAstar.

Southern hybridization. DNA fragments were transferred to Immobilon N (Millipore) according to the manufacturer's instructions, using a TransVac Blot apparatus (Hoefer) with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) as the transfer buffer. The DNA was cross-linked to the membrane by UV irradiation as specified by the instructions for the UV cross-linker apparatus.

Hybridization probes were generated by PCR using primers synthesized by the University of Illinois Biotechnology Center. The 2.1-kb *apeE*-specific probe was constructed by using primers apee3 (5′GCTCCATTATCTGCCTGT3′; bases 813 to 830) and apee17 (5′TAGCTCCAACTGACGAAAT3′; bases 2913 to 2931). The 429-bp probe specific for sequence upstream from *apeE* was constructed by first generating a 1.1-kb PCR product by using primers BamHIccw (5′CGAATTCATGCGTCCGGCGTAGA3′; located in vector sequence) and

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Genotype or description
Strains	
TN445	*apeA42*
TN478	*apeA42 apeR1*
TN925	*apeA42 apeR1 apeE*::Tn*5*
TN1379	*leuBCD485*
Plasmids	
pCM342	10-kb fragment inserted into *Bam*HI site of pBR328
pCM343	3.3-kb *Sau*3A fragment of pCM342 inserted into *Bam*HI site of pBR328

apee18 (5′CCGTTTCCGCCGACCCAGTGA3′; bases 1126 to 1106). This product was then cut with *Sma*I, and the 429-bp fragment (corresponding to bases 1 to 429) was purified by using a Geneclean II kit (Bio 101) according to manufacturer's instructions. Probes were labeled by random priming according to manufacturer's directions, using the Multiprime DNA labeling system (Amersham). Hybridization was carried out by using standard procedures (16) with 4× SSC in the prehybridization and hybridization solutions. Probes were hybridized overnight at 50°C and washed as follows: 1× SSC–1% SDS at room temperature for 1 min, 1× SSC–1% SDS at 50°C for 1 h, 0.5× SSC–1% SDS at 50°C for 1 h, and 0.1× SSC–0.2% SDS at 50°C for 1 h.

Preparation of cell extracts. Bacteria were grown to an optical density at 600 nm of 0.6 to 0.8 and harvested by low-speed centrifugation at 4°C for 10 min. The cell pellets were washed twice with 50 mM Tris-HCl (pH 7.5) (Tris buffer) and resuspended in 1/50 the original culture volume of Tris buffer. The cells were disrupted by sonication (Branson Sonifier 250, microtip) for 1 min and spun at 40,000 × *g* for 40 min at 4°C. The supernatant was kept as crude cell extract and stored at −70°C. SDS membrane extracts were prepared by resuspension of the pellet in 1/50 the original culture volume of 3% SDS in 50 mM phosphate buffer (pH 8.0), incubation at 100°C for 10 min, and centrifugation at 40,000 × *g* for 40 min at room temperature. The supernatant was removed and designated SDS-soluble membrane extract. Triton X-100 membrane extracts were prepared by extracting the pellet with 1/50 the original culture volume of 2% Triton X-100 in 50 mM phosphate buffer (pH 8.0) (phosphate buffer). After a 30-min incubation at room temperature, the extract was diluted in half by addition of an equal volume of phosphate buffer and spun at 40,000 × *g* at 4°C for 40 min. The supernatant was designated 1% Triton-soluble membrane extract and was used for all enzyme assays unless indicated otherwise. This procedure extracts about 25 to 35% of the NAPNE-hydrolyzing activity present in membranes of *apeR* mutant strains. Extracts of membranes prepared from strains carrying *apeE* mutations contained no esterase activity.

Enzyme assays. Hydrolysis of NAPNE was monitored spectrophotometrically essentially as described previously (20). The effects of inhibitors on hydrolysis rates were determined by mixing the inhibitor and enzyme, incubating the mixture for 30 min at room temperature, adding substrate, and monitoring the hydrolysis rate.

Gel electrophoresis. Six percent nondenaturing polyacrylamide 0.75-mm slab gels were run at 4°C according to the method of Davis (7) except that the stacking gel was omitted. The gels were run at 100 V until the tracking dye entered the gel and then at 250 V until the dye reached the end of the gel. The gels were soaked in deionized water for 30 min and then placed in 100 ml of NAPNE stain solution (90 ml of 0.1 M phosphate buffer [pH 6.8] with 10 mg of Fast Garnet GBC [Sigma] and 10 ml of NAPNE solution [0.2 mg/ml in *N*,*N*-dimethylformamide]). The gel was soaked in stain for 5 to 20 min until bands were apparent. The effects of inhibitors (except diisopropylfluorophospate [DFP]) on NAPNE hydrolysis after gel electrophoresis were determined by soaking the gel in the inhibitor solution for 30 min at room temperature and then staining as described above. For DFP, the enzyme was incubated with the inhibitor for 60 min at room temperature and the resulting gel was stained for activity.

Gels were subjected to SDS-PAGE according to the method of Laemmli (15), with the following modifications. Samples were suspended in SDS loading buffer lacking a reducing agent and heated to 55°C for 2 min before loading. After electrophoresis, gels were renatured by soaking in a solution of 1% glycerol–1% Triton X-100–50 mM Tris (pH 7.5) for 30 min. The gels were then stained as described above for nondenaturing gels.

Tris-Tricine SDS-polyacrylamide gels were run according to the method of Schagger and von Jagow (23). Samples were mixed with SDS sample buffer and incubated at 100°C for 10 min before loading. These gels were then stained with Coomassie blue.

Electroblotting. SDS-polyacrylamide gels were transferred to a ProBlot membrane (Applied Biosystems) with an Electroblot apparatus (Trans Blot) according to manufacturer's instructions, using 10 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS; pH 11.0)–10% methanol as a transfer buffer.

N-terminal sequencing. N-terminal sequence was determined by the University of Illinois Biotechnology Center, by using an Applied Biosystems (Perkin-Elmer) model 477A Protein/Peptide Sequencer with a model 120A on-line phenylthiodydantoin analyzer.

Nucleotide sequence accession number. The DNA sequence presented here is accessible from the GenBank database under accession no. AF047014.

RESULTS AND DISCUSSION

Cloning of the *apeE* locus. To isolate clones carrying the *apeE* gene, plasmids from a pBR328 chromosomal DNA library prepared from strain TN1379 (*apeA*$^+$ *apeE*$^+$ *apeR*$^+$) were transduced into TN445 (*apeA apeE*$^+$ *apeR*$^+$) and TN925 (*apeA apeE apeR*), and the resulting colonies were screened to identify those able to hydrolyze NAPNE. All NAPNE-hydrolyzing colonies were tested by using nondenaturing PAGE followed by NAPNE staining to determine whether they contained ApeA (protease I) or ApeE (see Materials and Methods). A single isolate containing ApeE was identified. This isolate contained plasmid pCM342, which was characterized further. This plasmid was found in the TN445 (*apeR*$^+$) background, and no plasmids carrying *apeE* were found in the *apeR* mutant strain. Subsequent experiments indicated that transfer of pCM342 to an *apeR* background produced small, slow-growing colonies, suggesting that this level of overproduction of ApeE is toxic. Restriction mapping showed that pCM342 contained an 8.3-kb insert. *Sau*3A partial digests of pCM342 were generated, cloned into pBR328, and transformed into *E. coli* DH5α. Screening these transformants for elevated NAPNE-hydrolyzing activity led to the isolation of pCM343, a plasmid with a 3.3-kb insert.

Nucleotide sequence of *apeE*. The entire insert DNA in pCM343 was sequenced. An additional 281 bp of the pCM342 insert immediately adjacent to this sequence was also determined, for a total of 3,536 bp. The sequence contained an open reading frame (ORF) consistent with the size predicted for *apeE*, based on SDS-PAGE of the ApeE enzymatic activity (approximately 60 kDa [5]). This ORF (bp 759 to 2729) predicts a 69.9-kDa protein. The predicted N-terminal amino acid sequence (positions 1 to 25) resembles a signal peptide, and since ApeE is a membrane-associated activity, we expected that the Ala-X-Ala sequence at amino acids 23 to 25 might serve as a signal peptidase cleavage site. This prediction was confirmed by N-terminal sequence analysis of the mature protein, which showed that it carries an N-terminal amino acid sequence beginning with amino acid 26. The predicted molecular mass of the mature protein (67.3 kDa) is somewhat larger than that estimated from SDS-PAGE (60 kDa). The C-terminal region of the protein conforms strikingly to the pattern noted for other outer membrane proteins (25) which contain hydrophobic amino acids at positions 1, 3, 5, 7, and 9 from their C termini. The C-terminal amino acid of ApeE is phenylalanine, and hydrophobic amino acids are located at positions 3, 5, 7, and 9 from the C terminus.

A search of GenBank using the BLAST program turned up another protein with strong similarity to ApeE. This protein (LipI; GenBank accession no. P40601) is an extracellular lipase from *P. luminescens*, an entomopathogenic member of the family *Enterobacteriaceae* (31). These predicted proteins show 41% amino acid sequence identity and 62% similarity (Fig. 1). The two proteins are approximately the same length (656 [*Salmonella*] and 645 [*Photorhabdus*] amino acids for the unprocessed proteins), and regions of identity and strong similarity extend throughout the sequences. An ORF of unknown function located between the *trpE* and the *trpGDC* genes of *Pseudomonas putida* (10) has a product that also shows significant similarity to ApeE. Alignment of the two sequences using the

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   ************************↓
 1 .MKRSFIFAPGMLALSISAISNAHAYNNLYVFGDSLSDGGNNGRYTVDGI 49
 1 MTQKRTLLKYGILSLALAAAPLSACAFDSLTVIGDSLSDTGNNGRWTWD.. 48

50 NGTESKLYNDFIAQQLGIELVNSKKGGTNYAAGGATAVADLNNKHNTQDQ 99
49 .SGQNKLYDEQLAERYGLELSPSSNGGSNYAAGGATATPELNPQDNTADQ 97

100 VMGYLASHSNRADHNGMYVHWIGGNDVDAALRNPADAQKIITESAMAASS 149
 98 VRQWLAKTGGKADHNGLYIHWVGGNDLAAAIAQPTMAQQIAGNSATSAAA 147

150 QVHALLNAGAGLVIVPTVPDVGMTPKIMEFVLSKG...GATSKDLAKIHA 196
148 QVGLLLDAGAGLVVVPNVPDISATPMLLEAVITAGLGAAAPPALKAALDA 197

197 VVNGYPTIDKDTRLQVIHGVFKQIGSDVSGGDAKKAEETTKQLIDGYNEL 246
198 LAEG.ATPDFASRQQAIRKALLAAAATVS.SNPFIQQLLVEQLLAGYEAA 245

247 SSNASKLVDNYNQLEDMALSQENGNIVRVDVNALLHEVIANPLRYGFLNT 296
246 AGQASALTDYYNQMEEKGLEQHGGNIARADINGLFKEILANPQAFGLTNT 295

297 IGYACAQGVNAGSCRSKDTGFDASKPFLFADDFHPTPEAHHIVSQYTVSV 346
296 VGMACPPGVSASACSSAMPGFNASQDYLFADHLHPGPQVHTIIAQYIQSI 345

347 LNAPYRVMLLTNANNVPVKGALASLDGRLQQLRNVDNEQGKLGVFGGYSG 396
346 IAAPVQATYLNQSVQSMAQGSRTTLDSRYQQLRQGENPVGSLGMFGGYSG 395

397 .............NHSHTLTLGSDYQIMDNILLGGMISRYQDNSSPADNF 433
396 GYQRYDNNEADGNGNHNNLTVGVDYQLNEQVLLGGLIAGSLDKQHPDDNY 445

434 HYDGRGYVFTAYGLWRYYDKGWISGDLHYLDMKYEDITRGIVLNDWLRKE 483
446 RYDARGFQAAVFSHLR.AGQAWLDSDLHFLSAKFSNIQRSITLGALRRVE 494

484 NASTSGHQWGGRITAGWDIPLTSAVTTSPIIQYAWDKSYVKGYRESGNNS 533
495 EGETNGRLWGARLTSGYDFVMVPWLTTGPMLQYAWDYSHVNGYSEKLNTS 544

534 TAMHFGEQRYDSQVGTLGWRLDTNFGYFNPYAEVRFNHQFGDKRYQIRSA 583
545 TSMRFGDQNAHSQVGSAGWRLDLRHSIIHSWAQINYRRQFGDDTYVANGG 594

584 INSTQTSFVSESQKQDTHWREYTIGMNAVITKDWGAFASISRNDGDVQNH 633
595 LKSTALTFSRDGKTQDKNWVDIAIGADFPLSATVSAFAGLSQTAGLSDGN 644

634 TYSFSLGVNASF 645
645 QTRYNVGFSARF 656
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FIG. 1. Comparison of *P. luminescens* lipase and *S. typhimurium* esterase amino acid sequences, generated by the GCG computer program Gap. The top sequence is that of the *P. luminescens lipI* (GenBank accession no. P40601) product, and the bottom sequence is that of the *S. typhimurium apeE* product. The row of asterisks above the sequence designates the signal peptide, and ↓ designates the first amino acid of the mature protein. The underlined sequence is the conserved motif characteristic of the GDSL family of esterases. A vertical line indicates sequence identity, a double dot represents very similar residues, and a single dot represents similar residues.

GCG Gap program revealed 49.5% similarity and 29% identity. A number of conserved regions could be identified by aligning ApeE, LipI, and the *Pseudomonas* ORF product. Although *E. coli* does not appear to have an *apeE* gene (see below), it does contain an ORF (YHJY_ECOLI; accession no. P37663) (24) whose product has significant similarity to an approximately 200-amino-acid region at the C terminus of ApeE.

Further analysis of the sequence carried by pCM343 revealed two additional ORFs (Fig. 2). One of these is in the opposite strand and upstream from *apeE* extending to the end of the region sequenced. This ORF shows strong similarity to *ybdG*, an uncharacterized ORF in *E. coli*. This *E. coli* ORF is located immediately downstream from *nfsB*/*nfnB* (encoding a nitroreductase) of *E. coli*. *Salmonella* contains a homolog of *nfsB*/*nfnB* which is situated at approximately the same place in the *Salmonella* chromosome as in that of *E. coli* (32). The *apeE* gene had been previously mapped by genetic methods to this region of the chromosome (6). The other ORF lies downstream from *apeE* and in the same strand. This ORF (*ybdI*) shows significant similarity to the *levR* gene of *Bacillus subtilis*, which encodes a transcriptional regulator of the levanase operon of this organism (9). LevR is a member of a family of regulators of σ^{54}-dependent promoters (26). No analog of this gene is found in *E. coli*. A comparison of the gene order in this region based on the *E. coli* and *Salmonella* sequences is shown in Fig. 2. It appears that both *apeE* and *ybdI* are part of a segment of DNA present in *Salmonella* but not in *E. coli* that is inserted (relative to *E. coli*) between the *Salmonella* homolog of *ybdG* and *nfsB*/*nfsN*. The inserted DNA extends at least to the *apeE*-distal end of the *Salmonella* DNA segment cloned into pCM342. This indicates that the insertion in *Salmonella* is at least 3 kb in length. Preliminary experiments in which a primer sequence taken from *ybdI* and another from the published *S. typhimurium nfnB* (32) sequence were used to PCR amplify *S. typhimurium* chromosomal DNA yielded an amplified fragment of approximately 6 kb. This implies that the insertion in *S. typhimurium* may be nearly 9 kb in length.

***apeE* is not present in *E. coli*.** Although mutations that lead to overexpression of ApeE are easily isolated as NAPNE-staining pseudorevertants of *S. typhimurium apeA* mutations, *E. coli apeA* mutants do not appear to give rise to NAPNE-staining pseudorevertants (5). This result combined with the observation that *apeE* might be contained on a DNA segment present in *Salmonella* but not *E. coli* suggested that *E. coli* may not have an *apeE* homolog. To test this, Southern blot analysis was carried out with a probe containing only *apeE* coding sequence and a probe carrying the *ybdG* ORF known to be present in both organisms. The *apeE*-specific probe hybridized to both *Salmonella* genomic DNA and DNA from the plasmid pCM343 but not to *E. coli* DNA (Fig. 3). A probe consisting of the 5′ ORF hybridized to chromosomal DNA from both *E. coli* and *S. typhimurium* (data not shown). We conclude that the *apeE* gene is not present in *E. coli* K-12. The sequence of the *E. coli* genome which appeared after this work was complete confirms that this organism does not contain an *apeE* homolog.

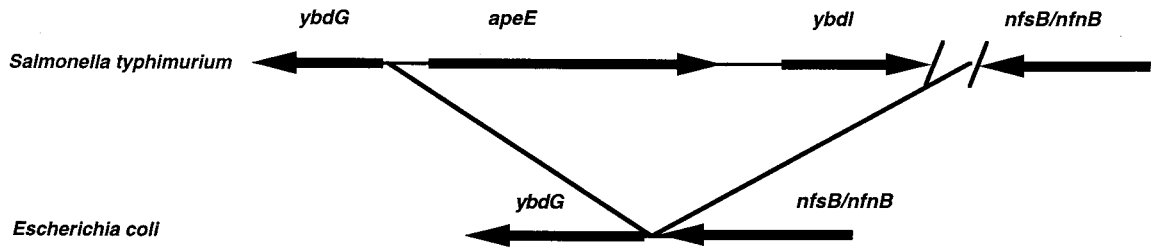

FIG. 2. Comparison of the gene order near *apeE* in *S. typhimurium* and *E. coli*. *apeE* and *ybdI* are part of a segment of DNA present in *Salmonella* but not in *E. coli* that is believed to be inserted between the *Salmonella* homolog of *E. coli ybdG* and *nfsB*/*nfsN*.

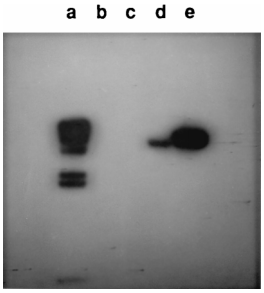

FIG. 3. Southern blot with *apeE* probe. DNA was digested with *Eco*RI, and hybridization was carried out with the *apeE*-specific probe described in Materials and Methods. Lanes: a, standards; b, *E. coli* DH5α; c, *P. luminescens*; d, *S. typhimurium*; e, pCM343.

Substrate specificity of *apeE*. The ApeE activity was originally recognized by its hydrolysis of NAPNE. We have previously reported evidence suggesting that ApeE does not have proteolytic activity despite the apparent specificity of the enzyme for the amino acid residue in the ester substrate (ApeE hydrolyzes the Phe ester but not the corresponding Leu substrate [6]). To learn more about the enzyme's specificity, we tested the ability of ApeE to hydrolyze a variety of chromogenic substrates (Table 2). Since none of the naphthylamides, nitroanilides, or peptides were hydrolyzed, we tentatively conclude that the enzyme will not cleave amide bonds. Clearly the enzyme is not specific for amino acid esters since naphthyl esters of short-chain fatty acids are rapidly cleaved. Indeed, the best substrates, naphthyl butyrate and naphthyl caproate, are hydrolyzed much more rapidly than the Phe derivatives. Many of the ester substrates that were not hydrolyzed are significantly more polar than those which were. Although naphthyl esters of lauric and palmitic acid were not hydrolyzed, *p*-nitrophenyl palmitate is a good substrate.

The *P. luminescens* lipase is able to hydrolyze Tween 80, a water-soluble oleic acid ester of a polyoxyalkylene derivative of sorbitan (31). A plate assay can be used to detect precipitation of liberated water-insoluble fatty acids, the product formed upon hydrolysis of Tween 80. This precipitation is visible as halos around the colonies. *Salmonella* strains TN1379, TN445, TN478, and TN925 were screened by using this plate assay for their abilities to hydrolyze Tween 80. After overnight incubation at 37°C, no precipitation was detectable with any of the *Salmonella* strains, but a *Pseudomonas aeruginosa* strain used as a positive control showed a large zone of precipitation. After overnight incubation at 4°C, however, a faint halo was apparent around *S. typhimurium* TN478 colonies but none of the other *Salmonella* strains. Since TN478 is the only strain in the group that overexpresses the ApeE esterase, this result suggests that ApeE cleaves Tween 80 but not as efficiently as the *P. luminescens* lipase. This could be due to a lower activity against this substrate or to the difference in localization of the two enzymes. The *Photorhabdus* enzyme is secreted into the culture medium, whereas the *Salmonella* activity is membrane bound.

TABLE 2. Substrate specificity of ApeE[a]

Type	Substrates
Hydrolyzed	β-Naphthyl esters of *N*-acetyl, *N*-benzoyl, and *N*-benzyloxycarbonyl Phe and of caproic acid; α- and β-naphthyl esters of acetic, propionic, and butyric acids; *p*-nitrophenyl esters of all straight-chain fatty acids C_6 to C_{16}
Not hydrolyzed	β-Naphthyl esters of *N*-acetyl Leu, *N*-methyl-*N*-toluene-*p*-sulfonyl Lys, and *N*-acetyl Arg and of lauric and palmitic acids; naphthol AS esters of propionic, phenylpropionic, and benzoic acids; *p*-nitrophenyl esters of *N*-acetyl Phe; β-naphthylamides of *N*-benzoyl Phe, Leu, Trp, and Leu-Phe; *p*-nitroanilide of *N*-acetyl Phe; peptides[b] PheAsp, AspPhe, PheGly, Phe_4, Phe_5, and PheGlyGly

[a] Based on qualitative assays using Triton X-100 membrane extracts as described in Materials and Methods. Membranes from a wild-type strain, an ApeR$^-$ strain overproducing ApeE, and an ApeE$^-$ strain were compared. In all cases in which hydrolysis was observed, the overproducer strain showed the most rapid color development and the ApeE$^-$ strain showed no activity. The most rapidly hydrolyzed compounds were the α- and β-naphthyl esters of butyric and caproic acids. Naphthol AS, 3-hydroxy-2-naphthoic acid anilide.

[b] Peptide hydrolysis was tested after electrophoresis of detergent extracts on nondenaturing polyacrylamide gels.

The deduced amino acid sequence of ApeE indicates that ApeE is a member of the GDSL family of ester hydrolases (28). This family of enzymes is characterized by an active site Ser residue located in most cases very close to the N terminus within the sequence GDSL (amino acids 33 to 36 in the *S. typhimurium* sequence of Fig. 1). This sequence differs from the GXSXG sequence found in most esterases, and the GDSL family appears to represent a distinct subfamily of serine hydrolases. The group also displays several other blocks of sequence similarity, including blocks that are thought to contain the Asp and His residues of the serine hydrolase catalytic triad (28). Although all act as esterases/lipases, the family appears to contain activities with distinct and varying specificities. Most members of the family hydrolyze a variety of ester substrates, however, and a full range of substrates has not been tested with all members of the family. It is interesting that the thioesterase product of the *tesA* (*apeA*) gene is also a member of this family although it is not similar to ApeE outside the blocks of similarity noted above. An arylesterase of *Vibrio mimicus* which is quite similar to TesA also contains the GDSL sequence (3). ApeE is most closely related to the lipase produced by *P. luminescens* and more distantly related to a protein encoded by a *Pseudomonas putida* ORF (10) and to a lipase/acyltransferase from *Aeromonas hydrophila* (27). The *Aeromonas* enzyme not only hydrolyzes soluble esters, neutral lipids, and phospholipids but also acts as a specific acyltransferase (13, 21, 27). Clearly a more detailed characterization of the substrate specificity of ApeE is in order. We do not know whether ApeE is activated by a lipid-water interface as are classical lipases, nor have we characterized its ability to hydrolyze neutral or phospholipid substrates. ApeE clearly does not belong to the class of lipases which require a lipid-water interface for activity (14, 29) since it hydrolyzes soluble esters such as NAPNE and β-naphthyl butyrate at an appreciable rate. The *apeE* gene designation seems clearly inappropriate since the acetyl-phenylalanine naphthyl ester esterase activity of the gene product almost certainly has no physiological significance. It seems reasonable to delay such a name change, however, until a better understanding of the enzyme's specificity and function allow assignment of a meaningful mnemonic.

Hydrolysis of MUCAP. One method for identifying *Salmonella* spp. in the clinical laboratory involves the use of methylumbelliferyl caprylate (MUCAP), a substrate that fluoresces upon hydrolysis of the ester bond (1, 8, 11, 22). This substrate can be used to distinguish salmonellae from other bacteria, including *Escherichia*, *Enterobacter*, *Yersinia*, and *Shigella* spp. Colonies of *Salmonella* strains TN1379, TN445, TN478, and TN925 were tested for their abilities to hydrolyze MUCAP. All *apeE*$^+$ strains fluoresced, but TN925, an *apeE* strain lacking the enzyme, did not fluoresce. In addition, *E. coli* DH5α containing plasmid pCM343 fluoresced, while the same *E. coli*

strain with pBR328, the parent vector for pCM343, did not. These results indicate that the *apeE* gene product is responsible for the hydrolysis of MUCAP in salmonellae.

Inhibitors of ApeE. The ability of various inhibitors to inhibit the hydrolysis of NAPNE by ApeE was tested either by spectrophotometric assays or by incubating the inhibitors with nondenaturing gels through which extracts containing the activity had been incubated prior to staining. Spectrophotometric assays showed that none of the following inhibitors had a significant effect (>20%) on ApeE activity: phenylmethylsulfonyl fluoride (3 mM), EDTA (0.1 M), *p*-chloromercuribenzoate (10 mM), iodoacetamide (1 mM), pepstatin A (0.25 mM), and β-mercaptoethanol (50 mM). In the gel activity stain assay, DFP (1 mM) showed strong inhibition although a faint band of activity could be observed after treatment with the inhibitor. We believe that this result indicates that ApeE is DFP sensitive. The residual activity may be a result of the failure of the inhibitor to fully inactivate the enzyme during the incubation time allowed, or it may represent a small fraction of the enzyme that is not sensitive to the inhibitor (2). Under the same conditions, none of the following had any observable effect: *N*-tosyl-L-lysine chloromethyl ketone (0.5 mM), *N*-tosyl-L-phenylalanine chloromethyl ketone (0.5 mM), eserine (1 mM), EDTA (100 mM), bis-*p*-nitrophenyl phosphate (1 mM), and *N*-ethylmaleimide (100 mM).

The ApeE esterase can be reactivated after SDS-PAGE. When a Triton X-100 extract of whole membranes containing ApeE is subjected to SDS-PAGE and the resulting gel is incubated in a renaturation buffer (see Materials and Methods), renatured enzyme is easily detected (data not shown). Using the more sensitive β-naphthyl caproate substrate, activity can be observed after electrophoresis of either boiled or unboiled samples. The electrophoretic mobility of the activity in the unboiled samples is somewhat greater than that observed after boiling, suggesting that the enzyme is not completely unfolded by treatment with SDS at room temperature.

Other enzymatic properties. When NAPNE was used as a substrate, the enzyme was found to have a pH optimum of approximately 8.0. Attempts to determine kinetic constants for this substrate were limited by its insolubility. No indication of saturation was observed at 0.15 mM NAPNE.

Physiological function of ApeE. The data that we present provide few clues concerning the physiological function of the ApeE protein. It is clearly not required for growth. It is conceivable that it is involved in the catabolism of fatty acid esters, although it is apparently not regulated by cyclic AMP receptor protein (based on the absence of a cyclic AMP receptor protein binding site in the promoter region). Preliminary experiments indicate that an *apeE*$^+$ strain but not an *apeE* mutant utilizes Tween 80 as a sole carbon source (6a). Perhaps elucidation of the nature of the regulatory gene which controls its transcription (*apeR*) will provide clues concerning ApeE's physiological role.

ACKNOWLEDGMENT

This work was supported by grant AI10333 from the National Institute for Allergy and Infectious Diseases.

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