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

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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 C6 to C16 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 ape 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 Sau3A partial digestion of DNA from S. typhimurium TN1379 and inserted into the BamHI 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 apeE:3′-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 pCMS42 were obtained by partial digestion of the plasmid with Sau3A; ligation into the BamHI site of pBR328, and electroporation into E. coli DH5α. The transformants were stained for activity as described above and restreaked. Although DH5α is apoA′, it is easy to distinguish the dark red color of a colony containing an apoE′ plasmid from the pink color of the parental colony. The presence of an apoE 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 ape-specific probe was constructed by using primers 5′-GCTTCAATTATGCTCGGTG3′; bases 813 to 830) and ape17 (5′-TACGGCTCAGTTCTGAAAT3′; bases 2913 to 2931). The 429-bp probe specific for sequence upstream from apeE was constructed by first generating a 1.3-kb PCR product by using primers BamHI (5′-GAAATTCTAGCTGTCGCGGCTAG3′; located in vector sequence) and
TABLE 1. Bacterial strains and plasmids used

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or description</th>
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<td>Strains</td>
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<td>TN445.............apeE42</td>
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<tr>
<td>TN445.............apeE42 apeR1</td>
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<tr>
<td>TN925.............apeE42 apeE::Tn5</td>
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<td>TN1379............leuB:C4D85</td>
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Plasmids

pCM342.............10-kb fragment inserted into BamHI site of pBR328
pCM343.............3.3-kb Sau3A fragment of pCM342 inserted into
BamHI site of pBR328

ape18 (5' CGCTTCCCCGCGACCAGTTA3'; bases 1126 to 1106). This product
was then cut with Smal, and the 429-bp fragment (corresponding to bases 1
to 429) was purified 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 (Amer-
sham). Hybridization was carried out using standard procedures (16) with 14
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 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 mix-
ture 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. After the dye reached the end of the gel, the gels were soaked in
demineralized water for 3 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 diisopropylfluorophospho-
ate [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 cut 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.

Trit-Tricin 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.

Electroelution. SDS-polyacrylamide gels were transferred to a ProBlot mem-
brane (Applied Biosystems) with an Electroblot apparatus (Trans Blot) accord-
ing to manufacturer’s instructions, using 10 ml 5-[3-cholamidopropyl]-dimeth-
ylammonium]-1-propanesulphonate (CHAPS; pH 11.0)–10% methanol as a transfer
buffer.

N-terminal sequencing. N-terminal sequence was determined by the Univer-
sity of Illinois Biotechnology Center, by using an Applied Biosystems (Perkin-
Elmer) model 477A Protein/Peptide Sequencer with a model 120A on-line phe-
nthyloxydantoin 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
transferred into TN445 (apeA + apeE + apeR) and TN925
(apeE + apeE + apeR), and the resulting colonies were screened to identify
those able to hydrolyze NAPNE. All NAPNE-hydro-
lyzing colonies were tested by using nondenaturing PAGE
followed by NAPNE staining to determine whether they con-
tained ApeA (protease I) or ApeE (see Materials and Meth-
ods). A single isolate containing ApeE was identified. This
isolate contained plasmid pCM342, which was characterized
further. This plasmid was found in the TN445 (apeR +) back-
ground, 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-grow-
ing colonies, suggesting that this level of overproduction of
ApeE is toxic. Restriction mapping showed that pCM342 con-
tained an 8.3-kb insert. Sau3A partial digests of pCM342 were
generated, cloned into pBR328, and transformed into E. coli
DH5a. 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 deter-
mined, 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) pre-
dicts 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 molec-
ular mass of the mature protein (67.3 kDa) is somewhat larger
than that estimated from SDS-PAGE (60 kDa). The C-termi-
nal 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
N-terminal. The C-terminal amino acid of ApeE is phenyla-
nine, 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
(Lip; 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 [Sal-
monella] and 645 [Pseudomonas putida] amino acids for the unproc-
essed proteins), and regions of identity and strong similarity
extend throughout the sequences. An ORF of unknown func-
tion located between the trpE and the trpGDC genes of Pseu-
domonas putida (10) has a product that also shows significant
similarity to ApeE. Alignment of the two sequences using the
E. coli, though aligning ApeE, LipI, and the tity. A number of conserved regions could be identified by GCG Gap program revealed 49.5% similarity and 29% identity. The row of asterisks above the sequence designates the signal peptide, and a single dot represents similar residues. A vertical line indicates sequence identity, a double dot represents very similar residues.

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 Salm onella but not in E. coli that is believed to be inserted between the Salmonella homolog of E. coli ybdG and nfsB/nfsN.
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 hydrolyzed. 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 $P.\ lumescens$ lipase is able to hydrolyze Tween 80, a water-soluble oleic acid ester of a polyoxylkylene 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 $37^\circ$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 $4^\circ$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 $P.\ lumescens$ 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.

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 GXXG 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 $P.\ lumescens$ 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).

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 $p$-nitrophenyl butyrate at an appreciable rate. The $apeE$ gene designation seems clearly inappropriate since the acetyl-phe nylalanine 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 $E.\ coli$ DH5α containing plasmid pCM343 fluoresced, while the same...
strain with pBR328, the parent vector for pCM343, did not. These results indicate that the apE gene product is responsible for the hydrolysis of MUCAP in salmonellae.

Inhibitors of ApE. The ability of various inhibitors to inhibit the hydrolysis of NAPNE by ApE was tested either by spectrophotometric assays or by incubating the inhibitors with non-denaturating 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 ApE activity: phenethylthiolsulfon 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 ApE 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 which 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 ApE esterase can be reactivated after SDS-PAGE. When a Triton X-100 extract of whole membranes containing ApE is subjected to SDS-PAGE and the resulting gel is incubated in a renaturation buffer (see Materials and Methods), ApE is subjected to SDS-PAGE and the resulting gel is in-

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 ApE. The data that we present provide few clues concerning the physiological function of the ApE protein. It is clearly not required for growth. It is conceiv-able 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 apE"- strain but not an apE 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 ApE's physiological role.

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REFERENCES


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nescens: cloning and sequencing of the lipase gene and analysis of its expres-