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Mutants of *Salmonella typhimurium* Deficient in an Endoprotease

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Three bands of hydrolytic activity toward the chromogenic protease substrate *N*-acetyl-DL-phenylalanine β-naphthyl ester (NAPNE) can be observed after gel electrophoresis of crude extracts of *Salmonella typhimurium* or *Escherichia coli*. Mutants deficient in one of these three activities have been isolated using a staining procedure that identifies colonies that show reduced ability to hydrolyze NAPNE. These mutants lack the strongest of the three bands of activity. The *Salmonella* mutations (designated *apeA*) are all co-transducible with *purE*, and the order *(pro)-apeA*-Hfr K17 origin-*purE* has been established. Strains carrying *apeA* mutations have wild-type doubling times. None of the *apeA* mutants isolated gains an auxotrophic requirement as a result of loss of the *apeA* gene product. The rates and extents of protein degradation during starvation for a carbon source or during growth after exposure to the amino acid analogue canavanine do not seem to be affected by *apeA* mutations. Revertants of *apeA* mutations (selected by screening for clones that have regained the ability to hydrolyze NAPNE) frequently contain a new enzymatic activity not found in wild-type cells.

Little is known about the role of individual proteases in the various proteolytic processes that take place in bacteria. It is known that bacteria are capable of extensive protein breakdown during starvation (14). Protein breakdown also takes place during normal growth with preferential degradation of misfolded, analogue-containing proteins (4, 14) and of protein fragments produced as a result of chain termination mutations (5). In addition to these processes that result in complete breakdown of proteins to amino acids, proteolytic processes can also generate large, sometimes enzymatically active protein fragments (1, 6, 7). It is not clear whether all these processes require the participation of the same set of enzymes. Possibly different families of proteolytic enzymes perform specific functions in the cell. There is some evidence, for example, that protein degradation during starvation may involve proteases different from those responsible for degradation during growth (15).

One major obstacle to the identification of the physiological roles of individual intracellular proteases in bacteria is that almost nothing is known about these enzymes. A chymotrypsin-like endoprotease from *Escherichia coli* has been studied in detail. Pacaud and Uriel (12) have shown that *E. coli* contains three electrophoretically separable enzymes capable of hydrolyzing the protease substrate *N*-acetyl-DL-phenylalanine β-naphthyl ester (NAPNE). One of these activities (protease I) has been purified and shown to possess proteolytic activity (6, 12). This enzyme is an endoprotease with unusual substrate and inhibitor specificity. It catalyzes the hydrolysis of NAPNE but is inactive toward *N*-acetyl-L-tyrosine ethyl ester. It attacks *E. coli* ribonucleic acid polymerase and polynucleotide phosphorylase but is only weakly active toward casein. The enzyme is sensitive to diisopropylfluorophosphate but is not inhibited by phenylmethane sulfonyl fluoride.

The isolation and characterization of mutants deficient in particular proteolytic enzymes is one appraoch to the study of the physiological roles of individual proteases. This paper reports (i) the isolation of mutant strains of *Salmonella typhimurium* and *E. coli* that lack protease I; (ii) the map position in *S. typhimurium* of the mutations that lead to the loss of this activity; and (iii) the effects of these mutations on growth and on protein degradation.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this work are listed in Table 1. All the *Salmonella* strains are derivatives of *S. typhimurium* LT2 except TN383, which is an LT7 derivative. *E. coli*

TABLE 1. *Bacterial strains*

Strain	Genotype	Origin
apeA1	*apeA1*	DES[a] induced in LT2
argF88	*argF88*	J. R. Roth
leu-485	*leu-485*	J. R. Roth
purE11	*purE11*	J. S. Gots
TN29	*leu-485 apeA4*	DES induced in *leu-485*
TN105	*leu-485 apeA7*	DES induced in *leu-485*
TN106	*leu-485 apeA8*	DES induced in *leu-485*
TN172	*argF88 apeA18*	DES induced in *argF88*
TN215	*leu-485 pepN10 pepA1 pepB1 pepD1*	Reference 10
TN247	*leu-485 pepN10 pepA1 pepD1 pepB1 apeA9*	DES induced in TN215
TN248	*leu-485 pepN10 pepA1 pepB1 pepD1 apeA10*	DES induced in TN215
TN256	*ara-7 leu-39 his-6857* Hfr K17	DES induced in SA965
TN316	*ara-7 his-6857* Hfr K17	Leu$^+$ transductant from TN256
TN352	*serA13 apeA16* Hfr K4	DES induced in SA534
TN353	*ara-7 his-6857 apeA17* Hfr K17	DES induced in TN316
TN383	*purE67 proAB47*	K. Sanderson
TN416	Wild type (isogenic with *purE11*)	Pur$^+$ transductant from *purE11*
TN442	*apeA21*	—[b]
TN443	*apeA22*	—[b]
TN444	*apeA39*	—[b]
TN445	*apeA42*	—[b]
TN446	*apeA43*	—[b]
TN447	*apeA44*	—[b]
TN478	*apeA42 apeD1*	DES induced in TN445
TN479	*apeA42 apeD2*	DES induced in TN445
TN482	*apeA42 apeD5*	DES induced in TN445
TN483	*apeA42 apeD6*	DES induced in TN445
TN488	*purE11 apeA47*	DES induced in *purE11*
SA965	*ara-7 leu-39* Hfr K17	K. Sanderson (19)
SA534	*serA13* Hfr K4	K. Sanderson (19)

[a] DES, Diethyl sulfate.

[b] These *apeA* mutations were all induced in LT2 with *N*-methyl-*N'*-nitro-*N*-nitrosguanidine. The mutations were then transferred to a common genetic background by P22 transduction, using strain *purE11* as recipient. Linkage data from these transduction crosses are presented in Table 3.

strain JC5029 (*ilv*$^-$ *thr*$^-$ *thi*$^-$ *spc*^{r} Hfr KL16) was obtained from S. D. Barbour.

Media and growth conditions. Media and growth conditions have been described previously (10). For the determination of doubling times, an exponentially growing culture was diluted into fresh medium to give an optical density at 600 nm of approximately 0.005, and the growth of the culture was followed by monitoring optical density at 600 nm in a Gilford spectrophotometer.

Mutagenesis. Mutagenesis with diethyl sulfate was carried out as described by Roth (17). Mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (Aldrich Chemical Co.) was performed as described by Miller (11).

Isolation of *apeA* mutants. Approximately 10^3 to 10^4 cells from a mutagenized culture were plated on nutrient agar and allowed to grow until small colonies were visible. The plate was then overlayed with soft agar. The presence in the colonies of NAPNE hydrolytic activity was detected by pouring a staining mixture over the plate. This mixture was prepared by adding 0.2 ml of a solution containing 10 mg of NAPNE (Sigma) per ml in dimethyl formamide to 5 ml of diazonium salt solution [pH 7.5; 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride, 8% (vol/vol) dimethyl formamide, and 10 mg of Fast Garnet GBC (Sigma)]. The plate was incubated at room temperature until most of the colonies acquired a red color (usually about 20 min). Weakly stained colonies were picked, single colony isolated twice, and retested for their staining properties by replica plating.

Hfr crosses. Plate mating of Hfr *apeA*$^+$ donors with marked F$^-$ *apeA*$^-$ recipients was performed by the method of Sanderson et al. (19).

Transduction. Transduction with phage P22 *int-4* was performed by the method described by Roth (17).

Preparation of extracts. Crude cell-free extracts were prepared as described previously (10).

Gel electrophoresis. Polyacrylamide gel electrophoresis was performed by the method of Davis (3) as previously described (10). Bands of NAPNE hydrolytic activity were located by immersing the gels after electrophoresis in the same reaction mixture as used for staining colonies. Increased contrast between activity bands and the gel background can be obtained by lowering the buffer concentration and/or the pH of the gel before staining. This can be done by soaking the gel in several changes of distilled water.

Assay of NAPNE hydrolytic activity. The hydrolysis of NAPNE by crude cell-free extracts was assayed using a procedure similar to that described by Pacaud and Uriel (12). A substrate solution containing 1.5×10^{-4} M NAPNE in 0.05 M phosphate buffer, pH 6.5, 10% (vol/vol) dimethyl formamide was equilibrated at 25 C in the cuvette chamber of a Gilford model 2000 recording spectrophotometer. The reaction was started by adding 10 to 50 μl of a cell-free extract (5 to 10 mg of protein per ml). The reaction was followed by recording the increase in absorbance at 328 nm. The initial velocity of the reaction was linear with protein concentration over the range of protein concentrations used. Specific activities were calculated by dividing the initial velocity by the protein concentrations. Protein concentrations were determined by the method of Lowry et al. (9), using crystalline bovine serum albumin as standard.

Protein degradation experiments. Carbon starvation-induced protein degradation was measured as follows. An overnight culture (grown in minimal glucose medium) was diluted to 5×10^7 cells/ml in minimal glucose medium containing 0.08 μCi of L-[4,5-^{3}H]leucine per ml (5 Ci/mmol; New England Nuclear Corp.). The culture was grown to a cell density of 2×10^8 to 5×10^8 cells/ml. The cells were pelleted by centrifugation, washed twice in minimal medium containing 300 μg of unlabeled L-leucine per ml, and resuspended in carbon-free medium also containing 300 μg of unlabeled L-leucine per ml. At intervals, 0.9-ml samples were withdrawn and added to 0.1 ml of 50% trichloroacetic acid. After

standing at room temperature for at least 30 min, the samples were centrifuged (10,000 × *g*, 4 C), and the supernatant solutions were clarified by a second centrifugation. Trichloroacetic acid-soluble radioactivity was determined by liquid scintillation counting in Triton-toluene scintillation fluid (13).

Canavanine-induced protein degradation was studied as follows. An overnight culture of the appropriate *arg*⁻ strain (grown in minimal glucose medium + 20 μg of arginine per ml) was diluted to 5 × 10^7 cells/ml in the same medium and grown to ~5 × 10^8 cells/ml. Cells were washed twice with minimal glucose arginine medium and resuspended in either the original medium or in medium containing 20 μg of canavanine per ml in place of arginine. After 12 min of incubation at 37 C, L-[4,5-^{3}H]leucine was added to each culture (final concentration, 0.2 μCi/ml). After an additional 5 min of incubation, each culture was rapidly filtered (membrane filter, 0.45-μm pore size; Millipore Corp.), washed, and resuspended in minimal glucose arginine medium containing 300 μg of unlabeled L-leucine per ml. The release of trichloroacetic acid-soluble radioactivity as a function of time was determined as described above.

RESULTS

Isolation of *apeA* mutants. When colonies of *S. typhimurium* LT2 on a nutrient agar plate were exposed to a mixture containing NAPNE and the diazonium salt Fast Garnet GBC, the colonies turned red in about 20 min. When a plate containing colonies of a mutagenized culture was stained in the same way, a few colonies (≈1 in 10^4) that stained a light orange color could be seen. Several such colonies were purified and retested for their altered staining properties. The mutations leading to the weak-staining phenotype were designated *apeA*.

Gel electrophoresis. When a crude cell-free extract of *S. typhimurium* LT2 was electrophoresed on a polyacrylamide gel and the gel was stained for hydrolytic activity toward NAPNE, three bands of activity were seen (Fig. 1b). When an extract of the mutant strain *apeA1* was electrophoresed and stained for activity, the slowest moving activity band (R_f ~0.2) was absent (Fig. 1a). All of the weakly staining mutants tested lost this band of activity but retained the other two bands.

Enzyme assay. The hydrolysis of NAPNE catalyzed by crude cell extracts can be followed spectrophotometrically at 328 nm (12). Under the assay conditions we have used (see Materials and Methods), the specific activities of all mutant strains tested were less than 10% of the specific activity observed in the wild-type strain. The data in Table 2 show the levels of NAPNE-hydrolyzing activity in a series of isogenic *apeA*⁻ strains. In these strains the *apeA*⁻

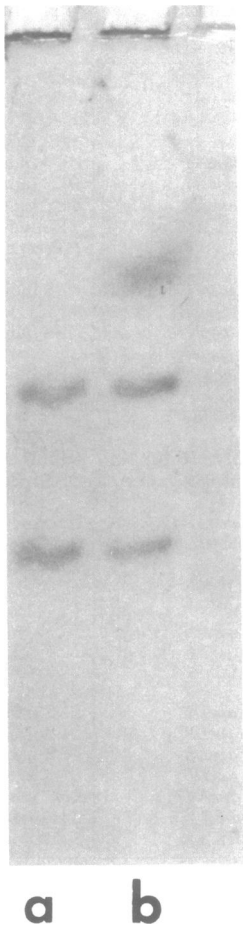


FIG. 1. *Polyacrylamide gel stained for hydrolytic activity toward NAPNE after electrophoresis of crude cell extracts of: (a) apeA1; (b) LT2 (wild type).*

TABLE 2. *Hydrolysis of NAPNE by extracts of mutant and wild-type strains*

Strain	Sp act	
	Absorbance units/min per mg of protein (× 10^3)	% of wild type
TN416 (wild type)	6.8	100
TN442	0.55	8.1
TN443	0.17	2.5
TN444	ND[a]	ND
TN445	ND	ND
TN446	0.32	4.7
TN447	0.42	6.2

[a] ND, Not detectable. We estimate that the sensitivity of the assay would not allow detection of specific activities below about 1 to 2% of wild-type levels.

alleles have all been transduced from the mutagenized background in which they were originally isolated into *purE11* (see below).

***E. coli apeA* mutants.** When a crude extract of *E. coli* strain JC5029 was electrophoresed on polyacrylamide and the gel was stained for NAPNE-hydrolyzing activity, a pattern identical to that found in *S. typhimurium* was observed. (This pattern of enzymatic activities in *E. coli* has been observed previously by Pacaud and Uriel [12].) Mutant derivatives of *E. coli* K strain JC5029 lacking the R_f ~0.2 enzyme have been isolated by the procedure described for isolation of the *Salmonella* mutants. The absence of the band of activity in the mutants has been verified by electrophoresis, but the strains have not been further studied.

Map position of *apeA* mutations. The map positions of the *apeA* mutations has been determined. Derivatives of strain TN29 *(leu-485 apeA4)* containing auxotrophic or fermentation markers were isolated after mutagenesis and penicillin selection or plating on indicator plates. These strains were crossed with appropriate Hfr's, prototrophic recombinants were selected, and these recombinants were scored for the presence of the donor *apeA*$^+$ allele. The results of these crosses suggested that the *apeA* mutation might lie in the region between 10 and 30 min on the *Salmonella* map. Other strains containing markers in this region were obtained, and *apeA* mutations were induced in them. These strains were crossed with appropriate Hfr's, prototrophic recombinants were selected, and the frequency of co-inheritance of the donor *apeA*$^+$ allele was scored. Data from these conjugation crosses showed that the *apeA4* mutation was approximately 85 to 90% linked to *purE*.

These results suggested that *apeA* might lie close enough to *purE* to be co-transduced with it by phage P22. The data in Table 3 demonstrate that these two markers are indeed co-transducible with a frequency of 1 to 5%. All of the *apeA* alleles tested were co-transducible with *purE*.

A strain isolated by Sanderson et al. (19) contains Hfr K17, which transfers chromosomal markers in a counterclockwise direction with a transfer origin located between *pro* and *purE*. The data in Table 4 show that Hfr K17 transfers *apeA* as an early marker and confirms Sanderson's observation that *purE* is transferred as a late marker. The results of the cross depicted in Table 4 (line 1) show that, when an Hfr K4 strain is used as donor, both *apeA* and *purE* are linked to *pro*. The results shown in line 2 indicate that, when an Hfr K17 strain is used as donor, *apeA* is linked to *pro* but *purE* is not. (The frequency of co-inheritance of *apeA* and *pro* is less in the cross using Hfr K17 than in the Hfr K4 cross. The frequency of recombination for very early markers in a conjugational cross is usually less than expected [8].) These results indicate that the relative orientation of *apeA* and *purE* must be *apeA*$^-$-Hfr K17 origin-*purE*. As expected if the F factor in Hfr K17 is inserted between *apeA* and *purE*, P22 lysates prepared on a strain containing Hfr K17 (TN353) are no longer capable of jointly transducing *ape* and *purE* (Table 3).

TABLE 3. *Transductional crosses*

Donor	Recipient	Selected marker	No. tested	% ApeA$^-$
apeA1	TN383 *(purE67)*	Pur$^+$	98	3
TN29 *(apeA4)*	TN383 *(purE67)*	Pur$^+$	100	3
TN105 *(apeA7)*	TN383 *(purE67)*	Pur$^+$	288	2
TN106 *(apeA8)*	TN383 *(purE67)*	Pur$^+$	288	1.4
TN247 *(apeA9)*	TN383 *(purE67)*	Pur$^+$	246	2
TN248 *(apeA10)*	TN383 *(purE67)*	Pur$^+$	98	5
TN353 *(apeA17* Hfr K17)	TN383 *(purE67)*	Pur$^+$	208	0[a]
apeA21[b]	*purE11*	Pur$^+$	155	1.9
apeA22[b]	*purE11*	Pur$^+$	91	2.2
apeA39[b]	*purE11*	Pur$^+$	110	1.0
apeA42[b]	*purE11*	Pur$^+$	48	2.1
apeA43[b]	*purE11*	Pur$^+$	166	1.8
apeA44[b]	*purE11*	Pur$^+$	152	1.3

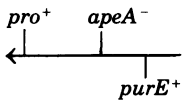
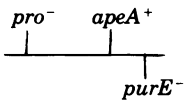
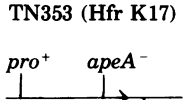
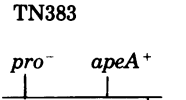
[a] In this strain, F is inserted between *apeA* and *purE* (see text).

[b] See footnote *b*, Table 1.

Growth properties of *apeA* strains. The function of the protease coded by the *apeA* gene is not known. Several potential protease functions might be required for normal growth of the cell. For example, certain enzymes may require proteolytic cleavage before they can function. Loss of a protease that normally performs this function might lead to auxotrophy if the enzyme requiring cleavage were part of a biosynthetic pathway, or to lethality if the enzyme were absolutely required for growth. In the latter case, strains with diminished protease levels (e.g., as a result of a leaky mutation) might still be isolatable, but some of these strains might show temperature-sensitive growth. To test these possibilities, we tested a series of *apeA*$^-$ strains isolated at 30 C for (i) auxotrophy at both 30 and 42 C, (ii) temperature sensitivity (i.e., ability to grow at 42 C), and (iii) sensitivity to phage P22. Phage sensitivity was tested on the chance that the missing protease might be required for phage maturation (e.g., see reference 7).

Twenty-one independent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced *apeA* mutations

TABLE 4. *Orientation of apeA and purE relative to the origin of Hfr K17*

Donor	Recipient	Selected marker	No. tested	Unselected marker	%
TN352 (Hfr K4)	TN383				
		Pro$^+$	128	ApeA$^-$ Pur$^+$	21 (59% were also Pur$^+$) 16 (80% were also ApeA$^-$)
		Pur$^+$	132	ApeA$^-$	83
TN353 (Hfr K17)	TN383				
		Pro$^+$	156	ApeA$^-$	11
				Pur$^+$	0

were isolated on nutrient agar at 30 C, and the absence of activity of the *apeA* gene product in each strain was verified by gel electrophoresis for each isolate. None of these mutations were temperature-sensitive lethals (i.e., all the isolates grew on nutrient agar at 42 C). Seven of these strains failed to grow on minimal medium. In all cases, however, it could be shown that the auxotrophy resulted from a secondary mutation and not from loss of the *apeA* gene product. Two additional strains were found to be resistant to phage P22. The transduction techniques used to show that the auxotrophic *apeA* strains contained more than one mutation could not be used with these phage-resistant strains. As a result, we have not been able to learn whether or not the phage resistance of these strains results from additional mutations unrelated to the *apeA* mutation. Since 7 of 21 strains carrying *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced *apeA* mutations also contained secondary mutations leading to auxotrophy and since, in a series of 20 *apeA*$^-$ mutants induced by diethyl sulfate, we have found no resistant clones, we believe it likely that loss of the *apeA* product does not result in either auxotrophy or phage resistance.

The growth rates of a series of strains carrying various *apeA*$^-$ alleles are shown in Table 5. Clearly, the *apeA* mutations do not affect the doubling times of these strains. Growth yields were also unaffected by *apeA* mutations.

Protein degradation in *apeA* strains. Protein degradation in *E. coli* (14) and in *S. typhimurium* (C. Yen and C. G. Miller, manuscript in preparation) is stimulated by starvation for carbon. The degradation of proteins formed during growth can be followed during starvation by measuring the release of trichloroacetic acid-soluble radioactivity from cells labeled during growth with radioactive leucine. Figure 2 shows that strain *apeA1* is able to degrade its protein during carbon starvation at approximately the same rate and to approximately the same extent as its wild-type parent.

TABLE 5. *Doubling times of wild-type and apeA*$^-$ *strains (minimal glucose medium)*

Strain	Doubling time (min)
TN416 (isogenic wild type)	48
TN442	49
TN443	48
TN444	47
TN445	47
TN446	48
TN447	48

Goldberg (4) has reported that, in *E. coli*, proteins synthesized in the presence of the arginine analogue canavanine are rapidly and completely degraded during growth. This process of growth degradation may not utilize the enzymes that are responsible for degradation during starvation (15). The effects of the *apeA1* mutation on analogue-induced degradation were therefore studied. Figure 3 shows that (i) in *Salmonella* (as in *E. coli*) proteins made in the presence of canavanine are rapidly degraded and (ii) the *apeA1* mutation does not significantly affect the rate of this process as measured by the release of trichloroacetic acid-soluble material from cells labeled with [^{3}H]-leucine while growing in the presence of canavanine.

Revertants of *apeA* mutations. Revertants of *apeA* mutations were isolated by staining a plate spread with colonies from a culture of a

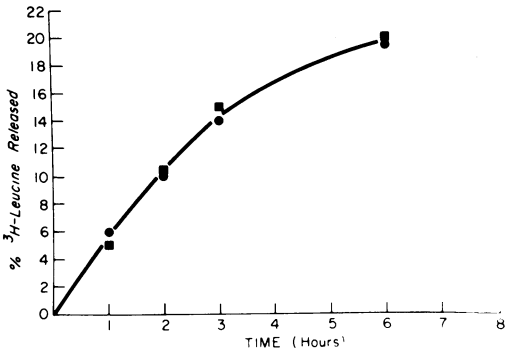


FIG. 2. *Protein degradation during starvation for a carbon source. Cultures of apeA1 (■) and LT2 (●) were labeled during growth with [³H]leucine and resuspended in carbon-free medium containing an excess unlabeled L-leucine. The trichloroacetic acid-soluble radioactivity released is expressed as a fraction of the total radioactivity incorporated (see Materials and Methods).*

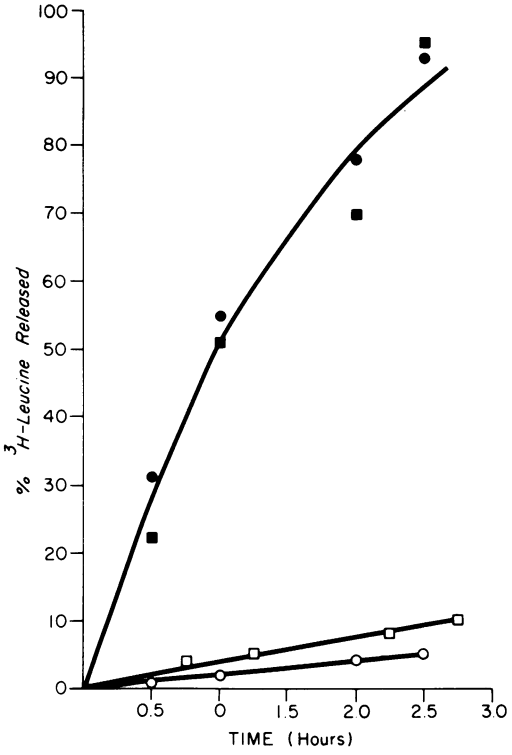


FIG. 3. *Canavanine-induced protein degradation. Degradation of proteins synthesized in the presence of canavanine was measured as described in Materials and Methods. Symbols: argF88 labeled during growth in canavanine (■) or in arginine (□); TN172 (argF88 apeA18) labeled during growth in canavanine (●) or in arginine (○).*

mutagenized *apeA*⁻ strain, using the procedure described for isolating *apeA*⁻ mutants. The red-staining revertants are easily identifiable and occur after diethyl sulfate mutagenesis at a frequency of about 1 in 10^4. Three to six independent revertants of four different *apeA*⁻ strains (TN442, -443, -444, and -445) were obtained. When crude extracts of these revertant strains were electrophoresed and the gels were stained for NAPNE-hydrolyzing activity, no band at the position expected for the *apeA* product was found in any of the extracts. Instead, a new activity with an R_f ~0.8 (the *apeA* enzyme moves at R_f ~0.2) was found in 25 to 75% of the red-staining revertants. To detect this new band, the amount of protein applied to the gel must be increased by about a factor of 2 over that used to detect the bands present in the wild-type extract. This band was never observed in wild-type or *apeA* mutant extracts even when high protein concentrations were applied to the gel.

DISCUSSION

The pattern of enzyme activities capable of hydrolyzing NAPNE detectable after polyacrylamide electrophoresis of a crude *S. typhimurium* extract is virtually identical to that observed for *E. coli* extracts by Pacaud and Uriel (12). The slowest moving enzyme always appears as a strong, broad band. The faster moving bands are sharper and weaker and vary in relative intensity in different extracts of the same strain. In all the mutants tested, the strong, slow moving band was absent, but both of the two faster moving bands were still present. The presence of these two activities in the mutants presumably accounts for the staining properties of mutant colonies: after long (>30 min) exposure to the substrate-dye mixture, *apeA* mutant colonies acquire a weak but distinct orange-pink color.

The absence of the *apeA*-coded enzyme seems to have no effect on cell growth. In addition, no pleiotropic effects (auxotrophy or temperature sensitivity) could be ascribed to *apeA* mutations. (The possibility that two *apeA* mutations might lead to P22 resistance is discussed in Results.)

The *apeA* mutations also have no effect on protein degradation as measured by the release of trichloroacetic acid-soluble radioactivity from labeled protein. Degradation during growth after exposure to the amino acid analogue canavanine and degradation during starvation for carbon were both tested and found not to be affected by *apeA* mutations. However,

the use of trichloroacetic acid-soluble radioactivity release as a measure of protein turnover may not have allowed detection of the role of the *apeA* gene product in one or both of these degradation processes. For example, in starvation-induced degradation neither the substrates for nor the pathway of degradation are known. It is conceivable that some proteins perhaps present in low amounts are degraded in an *apeA*$^+$ but not in an *apeA*$^-$ strain. It is also possible that other enzymes known to be present (see below) can substitute for the *apeA* product in the degradation pathway. The results reported in this paper do not, therefore, establish that the *apeA* product plays no role in protein degradation.

Although *apeA* mutations do not measurably affect degradation, other types of mutations leading to decreased protein degradation have been isolated. *Salmonella* mutations that lead to loss of peptidases have been shown to affect the rate and extent of carbon starvation-induced protein degradation (Yen and Miller, manuscript in preparation). Still other types of mutations isolated in *E. coli* diminish the rate of degradation during growth of polypeptide fragments produced as a result of chain termination mutations (2).

The *apeA* mutant strains retain not only the two faster moving bands of NAPNE-hydrolyzing activity, but also at least one other protease, a trypsin-like enzyme capable of hydrolyzing *N*-benzoyl-L-arginine *p*-nitroanilide and sensitive to the trypsin inhibitor *N*-α-tosyl-L-lysine chloromethyl ketone (TLCK). This enzyme has been observed in *E. coli* extracts (15) and in *Salmonella* extracts (C. Yen, Ph.D. thesis, Case Western Reserve University, Cleveland, Ohio, 1974) and is distinct from any of the NAPNE-hydrolyzing enzymes.

Another enzyme that might be able to function in place of the *apeA* gene product is the NAPNE-hydrolyzing activity present in "revertants" of *apeA* mutations. We do not know whether this enzyme is present in low amounts in the wild-type cell or what the nature of the mutations leading to its expression is. In addition, the ApeA$^-$ strains still contain at least eight peptidases (10). It is possible, therefore, that the remaining enzymes can substitute for the missing *apeA* product in protein degradation. It is also possible that the *apeA* product does not normally play a role in these processes. Pacaud and Uriel found that protease I is not very active toward casein and that its activity toward small-molecule substrates is severely restricted: *N*-acetylphenylalanine esters are hydrolyzed but *N*-acetyltyrosine esters are not. Also, the action of protease I on several macromolecular substrates seems to result in the production of high-molecular-weight products that retain either partial immunological or enzymatic activity (6). It may be, therefore, that the role of protease I in the cell is to perform relatively specific proteolytic modifications rather than to function in the more general protein breakdown observed after growth in the presence of an amino acid analogue or during starvation for a required nutrient. If this is the case, the ability of the ApeA$^-$ strains to grow normally must indicate that either other enzymes present in the cell can replace the *apeA* product or the proteolytic processes it normally performs are not necessary for normal cell growth.

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