

Peptidase-Deficient Mutants of *Escherichia coli*

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Received for publication 16 January 1978

Mutant derivatives of *Escherichia coli* K-12 deficient in several peptidases have been obtained. Mutants lacking a naphthylamidase, peptidase N, were isolated by screening for colonies unable to hydrolyze L-alanine β -naphthylamide. Other mutants were isolated using positive selections for resistance to valine peptides. Mutants lacking peptidase A, a broad-specificity aminopeptidase, were obtained by selection for resistance to L-valyl-L-leucine amide. Mutants lacking a dipeptidase, peptidase D, were isolated from a *pepN pepA* strain by selection for resistance to L-valyl-glycine. Starting with a *pepN pepA pepD* strain, selection for resistance to L-valyl-glycyl-glycine or several other valine peptides produced mutants deficient in another aminopeptidase, peptidase B. Mutants resistant to L-valyl-L-proline lack peptidase Q, an activity capable of rapid hydrolysis of X-proline dipeptides. Using these selection procedures, a strain (CM89) lacking five different peptidases has been isolated. Although still sensitive to valine, this strain is resistant to a variety of valine di- and tripeptides. The ability of this strain to use peptides as sources of amino acids is much more restricted than that of wild-type *E. coli* strains. Strains containing only one of the five peptidases missing in CM89 have been constructed by transduction. The peptide utilization profiles of these strains show that each of the five peptidases can function during growth in the catabolism of peptides.

The ability of *Escherichia coli* to utilize peptides as amino acid sources has been recognized for some time (18). Only recently, however, has there been any systematic study of the genetics of peptide utilization. Mutants of *E. coli* deficient in peptide uptake have been isolated and characterized (1, 4). Three *E. coli* mutants deficient in peptidases have also been described (5, 6, 20).

More extensive information is available concerning the genetics of *Salmonella typhimurium* peptidases. *Salmonella* strains containing mutations affecting three broad-specificity aminopeptidases (peptidase N, peptidase A, and peptidase B), a broad-specificity dipeptidase (peptidase D), and two proline-specific peptidases (peptidase Q and peptidase P) have been obtained (10, 13). The genes in which these mutations lie have been designated *pepN*, *pepA*, *pepB*, etc. The map positions of all of these loci except *pepB* have been determined (11). Because much of the biochemical and physiological data on peptide utilization was obtained with *E. coli* (12, 21), we thought it would be useful to isolate multiply peptidase-deficient strains of this organism. We have found that the pattern of peptidase activities present in soluble extracts of *E. coli* K-12 is similar to that of *Salmonella*. Procedures have been devised that allow posi-

tive selections for mutations affecting five of these enzymes. These procedures are, for the most part, different from those used to isolate *Salmonella* peptidase mutants.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this work are listed in Table 1. All are derivatives of *E. coli* K-12.

Media and growth conditions. Cultures were grown in nutrient broth (Difco) or LB medium (14). Vogel-Bonner E medium (23) or M9 medium (14), both containing 0.4% glucose and supplemented when necessary with 0.3 mM L-amino acids, were used as minimal media. All incubations were at 37°C.

Genetic techniques. Mutagenesis with 2-aminopurine (Aldrich Chemical Co.) was performed as described by Miller (14). Diethyl sulfate (Eastman Organic Chemicals) mutagenesis followed the procedure of Roth (17). Transduction using bacteriophage P1vir was carried out using standard procedures (14).

Selection of mutants. Mutants lacking peptidase N were isolated using L-alanine β -naphthylamide (ANA) as a chromogenic substrate to screen colonies as described previously (13). Mutants deficient in peptidase A were obtained from *pepN* parents by the following procedure: 0.1 ml of a $\frac{1}{20}$ dilution of an overnight culture (LB or nutrient broth) was plated on appropriately supplemented minimal medium (25 ml) onto which 0.1 ml of 0.1 M L-valyl-L-leucine amide (Val-Leu-NH₂) had been spread. After 48 to 72 h of

TABLE 1. Bacterial strains

Strain	Genotype	Source ^a
AB1157	<i>his leu pro arg thr thi str</i>	S. D. Barbour
TN165	<i>his leu pro arg thr thi str pepN1</i>	DES induced in AB1157
TN208	<i>his leu pro arg thr thi str pepN1 pepA1</i>	DES induced in TN165
TN827	<i>his leu pro arg thr thi str pepN1 pepA1 pepD1</i>	DES induced in TN208
CM10	<i>leu-9 Δ(pro-lac) met</i>	D. Zipser
CM17	<i>leu-9 Δ(pro-lac) met thyA</i>	Spontaneous <i>thy</i> from CM10
CM37	<i>leu-9 Δ(pro-lac) met thyA pepN102</i>	2-AP induced in CM17
CM48	<i>leu-9 Δ(pro-lac) met thyA pepN102 pepA11</i>	2-AP induced Val-Leu-NH ₂ ' in CM37
CM66	<i>leu-9 Δ(pro-lac) met thyA pepN102 pepA13</i>	Spontaneous Val-Leu-NH ₂ ' in CM37
CM86	<i>leu-9 Δ(pro-lac) met thyA pepN102 pepA11 pepB1</i>	Spontaneous Val ₃ ' from CM48
CM87	<i>leu-9 Δ(pro-lac) met thyA pepN102 pepA11 pepB2</i>	Spontaneous Val-Leu' from CM48
CM88	<i>leu-9 Δ(pro-lac) met thyA pepN102 pepA11 pepB3</i>	Spontaneous Val-Val' from CM48
CM89	<i>leu-9 Δ(pro-lac) met thyA pepN102 pepA11 pepB1 pepQ10</i>	Spontaneous Val-Pro' from CM86
CM91	<i>leu-9 Δ(pro-lac) met thyA pepQ12</i>	Spontaneous Val-Pro' from CM17
CM92	<i>leu-9 Δ(pro-lac) met thyA pepN102 pepA13 pepB4</i>	Spontaneous Val-Val' from CM66
CM103	<i>leu-9 Δ(pro-lac) met thyA pepA11 pepB1 pepQ10</i>	P1 transduction: CM17 × CM89
CM104	<i>leu-9 Δ(pro-lac) met thyA pepN102 pepB1 pepQ10</i>	P1 transduction: CM17 × CM89
CM105	<i>leu-9 Δ(pro-lac) met thyA pepA11 pepN102 pepQ10</i>	P1 transduction: CM17 × CM89
CM106	<i>leu-9 Δ(pro-lac) met thyA pepA11 pepN102 pepB1</i>	P1 transduction: CM17 × CM89
CM107	<i>leu-9 met thyA pepA11 pepB1 pepQ10 pepN102</i>	P1 transduction: <i>lacZ521</i> × CM89

^a DES, Diethyl sulfate; 2-AP, 2-aminopurine.

incubation at 37°C, colonies were picked, purified, and replica plated to test for sensitivity to valine (10⁻⁴M) and to Val-Leu-NH₂. Independent clones that were sensitive to valine but resistant to Val-Leu-NH₂ were saved. Mutants lacking peptidase D were isolated from a *pepN pepA* derivative of AB1157 mutagenized with diethyl sulfate by plating a 1/20 dilution of an overnight nutrient broth culture on appropriately supplemented minimal plates spread with 0.1 ml of 0.1 M L-valyl-glycine (Val-Gly). Again, purified resistant clones were tested by replica plating for valine sensitivity. (All strains derived from CM17 are *pepD* because the *pro lac* deletion in this strain includes the *pepD* locus [see below].) Mutations affecting peptidase B were isolated by plating a 1/20 dilution of a *pepN pepA pepD* strain on an appropriately supplemented minimal plate spread with 0.1 ml of a 0.05 M solution of either L-valyl-L-leucine (Val-Leu), L-valyl-L-valine (Val-Val), or L-valyl-L-valyl-L-valine (Val₃). *pepB* strains arise as mutants resistant to any one of these peptides. Mutants lacking peptidase Q were isolated on minimal glucose plates spread with 0.1 ml 0.1 M L-valyl-L-proline (Val-Pro). In all cases the absence of the peptidase from the valine-sensitive, valine peptide-resistant clones was confirmed by electrophoresis and peptidase activity staining of crude extracts of the mutant strains. In most selections, 10 to 30% of the clones selected for resistance to the valine peptide were valine sensitive.

Utilization of and sensitivity to peptides. Strains were tested for sensitivity to valine and valine peptides and for utilization of other amino acids and peptides as amino acid sources by spotting 5 to 10 μl of peptide solution (0.025 to 0.05 M) onto an appropriately supplemented minimal plate over which a soft agar overlay containing 0.1 to 0.2 ml of the strain to be tested had been poured. (Neither CM17 nor CM89 growing on leucine-supplemented plates was inhibited by Leu-Gly or Leu-Gly-Gly under these conditions.)

Results were scored after overnight incubation. Peptides were obtained from commercial sources (Cyclo Chemical Co.; Sigma; Bachem). All peptides used contain only L-amino acid residues (or glycine).

Gel electrophoresis and peptidase activity stain. Cell extracts were prepared from stationary-phase cells as described previously (13). Electrophoresis in nondenaturing polyacrylamide gels was performed according to Davis (3), omitting stacking and sample gels. Usually slab rather than cylindrical gels were used. Peptidase activities were detected on the gels using the procedure of Lewis and Harris (9) as described previously (13). In some experiments, gels were preincubated for 30 min in 0.1 mM solutions of CoCl₂ in 0.1 M phosphate buffer (pH 7.5). MnCl₂ was omitted from the activity stain mixture when staining these preincubated gels. Electrophoretic mobilities of activity bands are expressed as *R_f*, the distance from the origin travelled by the band, divided by the distance moved by the tracking dye front.

RESULTS

***E. coli* peptidases.** Figure 1 (well a) shows the pattern of Leu-Gly-hydrolyzing peptidase activities detected after electrophoresis of a crude extract of *E. coli* K-12 strain CM17. By using other peptide substrates, the specificities of the peptidase activities present could be qualitatively determined. Data from such experiments are presented in Table 2. We have tentatively used the same designations for these *E. coli* activities that have been used previously for peptidases present in *S. typhimurium* extracts (10, 13). The following activities have been observed on these gels. (Activities are assigned the same numbers as in Table 2. Activities 4, 5, and 6 are not present in Fig. 1 because they do not

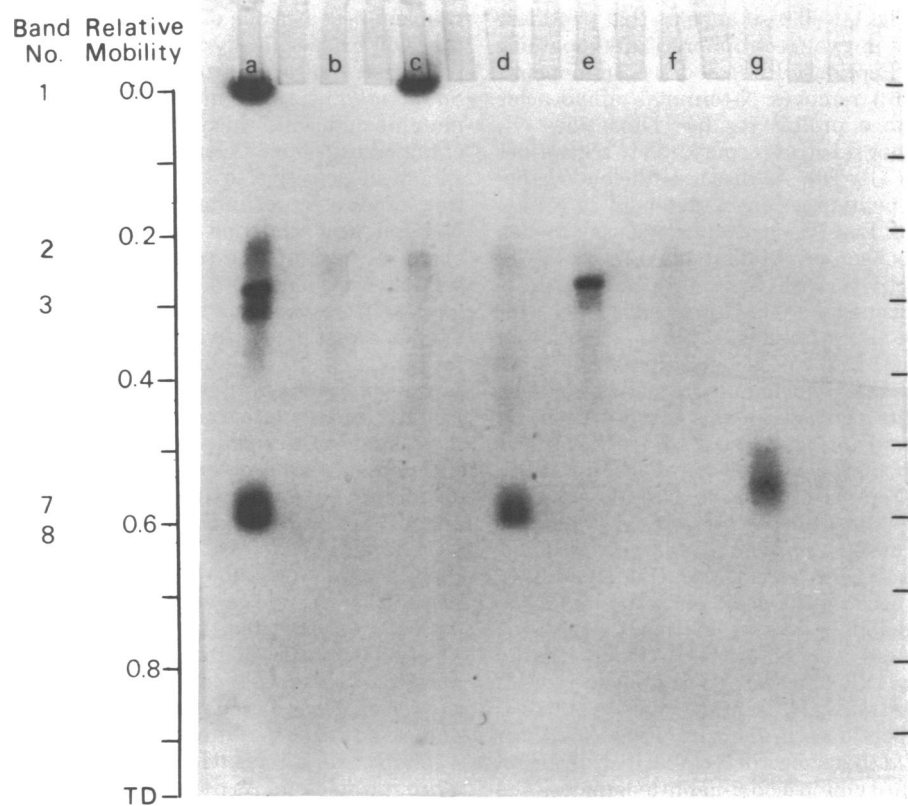


FIG. 1. *Leu-Gly*-hydrolyzing peptidase activities detectable after gel electrophoresis. A 3- to 5- μ l sample of cell extract (8 to 10 mg of protein per ml) was subjected to electrophoresis in a polyacrylamide gel and stained for peptidase activity after preincubation of the gel slab in 1 mM CoCl_2 using *Leu-Gly* as substrate. Wells: (a) CM17 (*pepD*); (b) CM89 (*pepD*, *N*, *A*, *B*, *Q*); (c) CM104 (*pepA*⁺ derivative of CM89); (d) CM103 (*pepN*⁺ derivative of CM89); (e) CM105 (*pepB*⁺ derivative of CM89); (f) CM106 (*pepQ*⁺ derivative of CM89); (g) CM107 (*pepD*⁺ derivative of CM89). Bands: (1) peptidase A; (2) $R_f \sim 0.28$ peptidase; (3) peptidase B; (7) peptidase D; (8) peptidase N. The band numbers correspond to those of the Results section of the text and to Table 2. TD, Tracking dye front.

TABLE 2. Substrate specificity patterns of peptidase activities

Band no.	R_f	Designation	Substrates			
			Leu-Gly	Leu-Gly-Gly	Leu-Pro	ANA
1	0	Peptidase A	+	+	-	-
2	0.28		\pm^a	+	-	-
3	0.3	Peptidase B	+	+	-	-
4	0.41	Peptidase P	-	-	\pm^b	-
5	0.47		-	$+\text{c}$	-	-
6	0.51	Peptidase Q	-	-	+	-
7	0.55	Peptidase D	+	-	-	-
8	0.6	Peptidase N	+	+	-	+

^a Activity toward *Leu-Gly* is usually not observed unless the gel is preincubated in CoCl_2 .

^b See text.

^c Variably present. Stimulated by preincubation in CoCl_2 .

hydrolyze *Leu-Gly*.) (1) Peptidase A ($R_f \sim 0$) is a broad-specificity aminopeptidase. This enzyme is almost certainly the same as that purified from *E. coli* by Vogt (13, 24). (2) A band of activity at $R_f \sim 0.28$ is variably present. Preincubation of the gels in 0.1 mM CoCl_2 increases the strength of this band so that hydrolysis of both *Leu-Gly* and *Leu-Gly-Gly* can usually be observed. In the absence of preincubation in CoCl_2 , the *Leu-Gly* activity of this band is frequently not detected. No mutants lacking this enzyme have been isolated. (3) Peptidase B ($R_f \sim 0.3$) is sometimes observed (using either *Leu-Gly* or *Leu-Gly-Gly*) as a single, broad band of activity. In other gels (such as that pictured in Fig. 1) two distinct activity bands are seen. Since *pepB* mutants lose both bands (Fig. 1, well b)

and *pepB*⁺ transductants regain both simultaneously (Fig. 1, well e), it appears that peptidase B can exist in two electrophoretically separable forms. (4) Peptidase P (*R_f* ~ 0.41) (aminopeptidase P [26]) removes N-terminal amino acids adjacent to a proline residue. Dipeptides (X-Pro) are poor substrates compared to tripeptides (X-Pro-Y) (10, 26). As discussed below, we believe this peptidase can be detected as a relatively weak Leu-Pro-hydrolyzing activity when the gel has been preincubated in 0.1 mM CoCl₂. (5) An activity with *R_f* ~ 0.47 is sometimes detected using Leu-Gly-Gly as substrate. This activity is also stimulated by preincubation of the gel in 0.1 mM CoCl₂, but differs from the *R_f* ~ 0.28 enzyme in its inability to attack Leu-Gly. No mutants deficient in this activity have yet been isolated. (6) Peptidase Q (*R_f* ~ 0.51) attacks X-Pro dipeptides such as Leu-Pro or Val-Pro (13). (7) Peptidase D (*R_f* ~ 0.57) is a broad-specificity dipeptidase present in wild-type strains of both *E. coli* and *S. typhimurium*. This enzyme is absent from CM17 (Fig. 1, well a), however, because the *pepD* locus lies under the *pro lac* deletion present in this strain. Pro⁺ recombinants isolated from CM17 do contain peptidase D (Fig. 1, well g), as do wild-type *E. coli* K-12 strains. (8) Peptidase N (*R_f* ~ 0.6) is the only peptidase present in either *E. coli* or *Salmonella* that shows detectable hydrolytic activity toward amino acid β-naphthylamides. The data of Table 2 combined with the properties of the mutant strains described below suggest that each of these activities is a distinct entity reasonably well defined by a characteristic electrophoretic mobility and substrate specificity pattern.

Isolation of peptidase mutants. *E. coli* K-12 is sensitive to valine (22) and to valine-containing peptides (4). Such valine peptides have been used to select mutants deficient in peptide uptake (4). If *E. coli* is sensitive to these peptides only because valine can be released from them and not because the peptides themselves are toxic, valine-containing peptides should also be useful in selecting peptidase mutants. Since several different peptidases present in the cell can

usually hydrolyze a given peptide (10, 13), the problem becomes one of finding valine peptides that will be specifically hydrolyzed by only one peptidase. Reasoning from the properties of the *Salmonella* peptidase mutants, we thought that mutants deficient in peptidase N (which can be obtained using an efficient screening procedure) should be sensitive to valine-containing dipeptide amides. *E. coli* mutants lacking peptidase N were easily obtained using ANA as a histochemical reagent in a screening procedure described previously (13). These *pepN* mutants lack the fastest-moving band of peptidase activity but are still sensitive to all valine peptides tested (Table 3), including Val-Leu-NH₂. Spontaneous mutants resistant to Val-Leu-NH₂ were isolated (see Materials and Methods) and tested for sensitivity to valine. (Mutants resistant to the peptide because they cannot hydrolyze it should still be sensitive to valine.) Strains resistant to Val-Leu-NH₂ but still sensitive to valine were found. Crude cell extracts prepared from these strains were subjected to electrophoresis in a polyacrylamide gel and stained for peptidase activity. As expected, all such isolates tested (9/9) showed loss of the enzyme activity that does not enter the gel (peptidase A). We followed the genetic locus designation used in *Salmonella* and called these mutations *pepA*.

The *E. coli* strains derived from CM17 carry a *pro lac* deletion. As noted above, this deletion includes the *pepD* gene, which is known to map near *pro* in both *Salmonella* and *E. coli* (13). *pepN pepA* strains derived from CM17 therefore lack three peptidases: peptidase N, peptidase A, and peptidase, D, a broad-specificity dipeptidase. Mutations lacking peptidase D can be isolated from a *pepN pepA pepD*⁺ strain by selecting for resistance to Val-Gly. Such mutants (for example, TN827) have been isolated from a *pepN pepA* derivative of *E. coli* AB1157.

The pattern of valine peptide sensitivities of the mutants described above can be seen in Table 3. Since strains missing peptidases N, A, and D are sensitive to Val-Leu, Val-Val, and Val₃, we set out to isolate strains resistant to each one of these peptides in the hope that

TABLE 3. Sensitivities of *pep* mutants to valine and valine peptides^a

Strain	Peptidase genotype	V	VLA	VG	VV	VL	VP	VGG	VVV
CM17	<i>pepD</i>	S	S	S	S	S	S	S	S
CM37	<i>pepD pepN</i>	S	S	S	S	S	S	S	S
CM66 ^b	<i>pepD pepN pepA</i>	S	R	R	S	S	S	R	S
CM92	<i>pepD pepN pepA pepB</i>	S	R	R	R	R	S	R	R
CM89	<i>pepD pepN pepA pepB pepQ</i>	S	R	R	R	R	R	R	R
CM91	<i>pepD pepQ</i>	S	S	S	S	S	R	S	S

^a V, Valine; VLA, Val-Leu-NH₂; VG, Val-Gly; VV, Val-Val; VL, Val-Leu; VP, Val-Pro; VGG, Val-Gly-Gly; VVV, Val₃. S, Sensitive; R, resistant.

^b The *pepD pepN pepA* derivative of AB1157 (TN827) shows the same pattern of sensitivities as CM66.

mutants lacking another peptidase would be obtained. Spontaneous mutants resistant to Val-Leu, Val-Val, or Val₃ were obtained (Materials and Methods). Many of these mutants were still sensitive to valine. When cell extracts of these strains were tested for the presence of peptidase activity after electrophoresis, several were found to lack peptidase B. Selection for resistance to each one of the three peptides (Val-Leu [CM87], Val-Val [CM88 and CM92], and Val₃ [CM86]) gave rise to *pepB* mutants. The pattern of valine peptide sensitivities is the same for all of these mutants and is given in line 4 of Table 3. As expected, mutants selected as resistant to any one of these three peptides are resistant to the other two.

In addition to the broad-specificity enzymes described above, both *E. coli* and *Salmonella* contain peptidases specific for proline peptides. One of these enzymes, peptidase Q, seems to be an X-Pro-specific dipeptidase (10). The other enzyme, peptidase P, can hydrolyze X-Pro dipeptides but much less efficiently than peptidase

Q, preferring larger peptides of the type X-Pro-Y (10, 26). Since L-valyl-L-proline (Val-Pro) is a substrate for peptidase Q in *Salmonella* and since *E. coli* K-12 is sensitive to this peptide, we thought that mutants resistant to Val-Pro might lack peptidase Q. Such mutants (Val-Pro resistant, Val sensitive) were easily isolated. After electrophoresis, crude cell extracts showed no detectable band of Val-Pro-hydrolyzing activity. Activity toward Leu-Pro ($R_f \sim 0.41$) could be seen, however, when the gel was preincubated in buffer containing Co^{2+} (Fig. 2B). These mutants grew slowly on Leu-Pro as a proline source (the parent strain grows well), but as well as the parent on Gly-Pro-Ala. This is precisely the phenotype of *Salmonella* mutants that are *pepQ pepP*⁺ (10). The most likely explanation of the properties of these *E. coli* mutants is that they have lost peptidase Q but retain peptidase P. Using the Val-Pro selection, spontaneous *pepQ* mutations could be introduced into strains already lacking peptidases N, A, B, and D. Strain CM89 is an example of such a strain lacking five

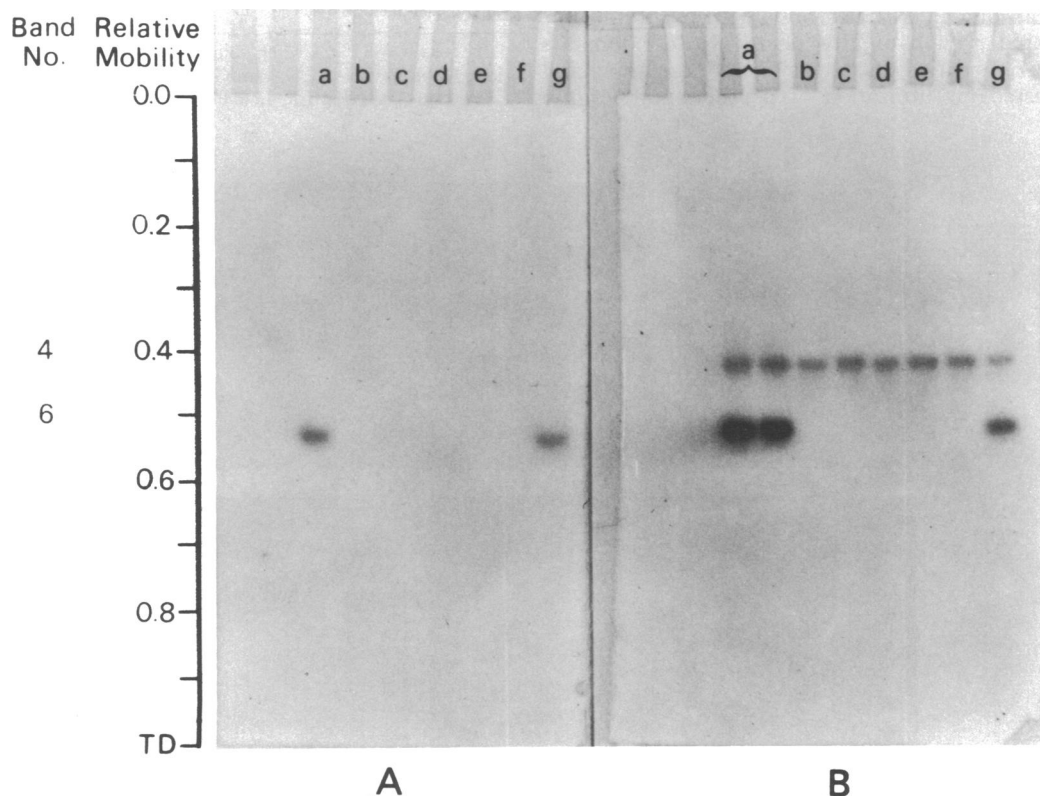


FIG. 2. Leu-Pro-hydrolyzing peptidase activities detectable after gel electrophoresis. A 3- to 5- μ l sample of cell extract (8 to 10 mg of protein per ml) was subjected to electrophoresis in a polyacrylamide gel and stained for peptidase activity using Leu-Pro as substrate. Wells: (a) CM17 (*pepD*); (b) CM89 (*pepD*, *N*, *A*, *B*, *Q*); (c) CM103 (*pepN*⁺ derivative of CM89); (d) CM104 (*pepA*⁺ derivative of CM89); (e) CM105 (*pepB*⁺ derivative of CM89); (f) CM107 (*pepD*⁺ derivative of CM89); (g) CM106 (*pepQ*⁺ derivative of CM89). TD, Tracking dye front. (A) Gel preincubated in buffer; (B) gel preincubated in CoCl_2 (see the text).

peptidases (N, A, B, D, and Q). The absence of peptidases A, B, and N in CM89 is shown in Fig. 1, well b, and the absence of strong Leu-Pro-hydrolyzing activity is demonstrated in Fig. 2A, well b. The pattern of resistance to valine peptides of CM89 is shown in Table 3. Mutants missing peptidase Q retain sensitivity to the other valine peptides, as shown in line 6 of Table 3. The valine peptide sensitivities of a series of peptidase-deficient strains derived from CM17 are shown in Fig. 3.

Introduction of wild-type *pep* alleles into CM89 by transduction. If each successive step in the construction of CM89 involved only a single mutational event, leading to the loss of a single peptidase, it should be possible to build by transduction strains containing only one of the five peptidases missing in CM89. Using CM17 as a donor and CM89 as recipient, P1 transductional crosses were carried out. Recombinants able to use Leu-Gly as a leucine source were selected in one cross and Leu-Pro-utilizing recombinants in another. (Strains that are *pepQ pepP*⁺ grow so poorly on Leu-Pro that *pepQ*⁺ transductants can be obtained by selection for rapid growth on this peptide.) Recombinants were purified by single colony isolation and tested for utilization of peptides and for the

ability to hydrolyze ANA. All Leu-Gly-utilizing recombinants fell into three classes (Table 4). Crude extracts were prepared from a representative of each class, and the peptidases present in these extracts were identified after gel electrophoresis. CM104 (class I) regains only peptidase A, CM103 (class II) only peptidase N, and CM105 (class III) only peptidase B. (See Fig. 1, wells c, d, and e.)

Since CM17 is *pepD*, no *pepD*⁺ recombinants can be isolated from crosses using CM17 as a donor. To construct a *pepD*⁺ strain from CM89, *lacZ521* (a *lac* point mutation) was used as donor and Pro⁺ transductants were isolated. These Pro⁺ transductants all acquired the ability to use Leu-Gly as a leucine source, and one of them, CM107, was shown (by electrophoresis of

TABLE 4. Peptide utilization patterns of recombinants^a

Recombinant class	LG	LGG	LAA	LP	ANA
I (CM104)	+	+	+	-	-
II (CM103)	+	+	+	-	+
III (CM105)	+	+	-	-	-

^a Cross: CM17 × CM89; selection: Leu-Gly utilization. Abbreviations: LG, Leu-Gly; LGG, Leu-Gly-Gly; LAA, Leu-Ala-NH₂; LP, Leu-Pro.

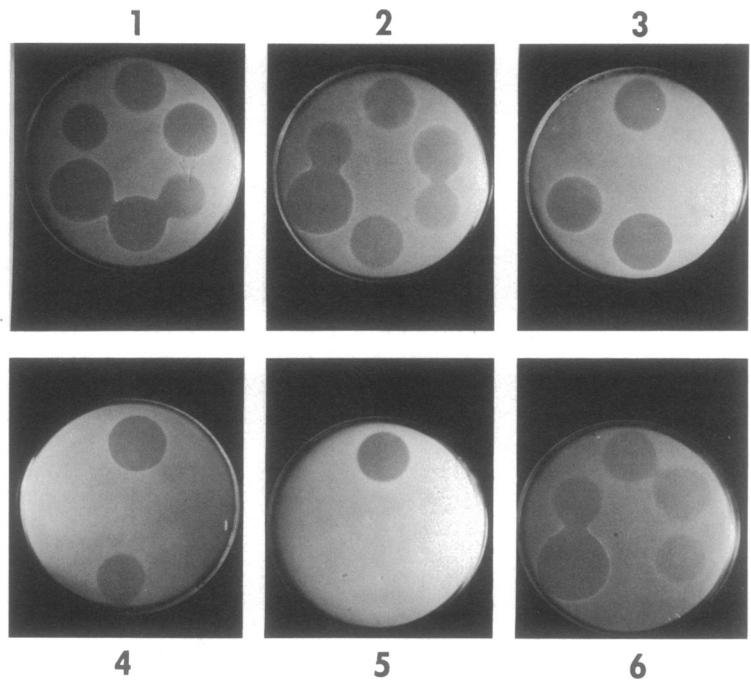


FIG. 3. Patterns of sensitivity to valine and valine peptides. A 10- μ l sample of 0.025 M peptide or amino acid was spotted on the surface of a soft agar overlay containing 0.2 ml of overnight cultures. Inhibitors in each plate are (reading clockwise from the top) valine, Val-Leu-NH₂, Val-Gly, Val-Pro, Val₃, Val-Gly-Gly. Strains are (1) CM17 (*pepD* parent), (2) CM37 (*pepD pepN*), (3) CM48 (*pepD pepN pepA*), (4) CM86 (*pepD pepN pepA pepB*), (5) CM89 (*pepD pepN pepA pepB pepQ*), (6) CM91 (*pepQ*).

cell extract followed by activity stain) to have regained only peptidase D (see Fig. 1, well f). This observation confirms previous results indicating that the map position of *pepD* in *E. coli* is approximately the same as in *Salmonella* (13).

A *pepQ*⁺ derivative of CM89 was isolated from a transduction cross with CM17 as donor. Selection for use of Leu-Pro as a leucine source gave only one type of recombinant. These recombinants all grew on Leu-Pro but not on Leu-Gly or Leu-Gly-Gly. They did not hydrolyze ANA. Electrophoresis of one of them (CM106) showed that peptidase Q was present (see Fig. 2A, well g). As shown in Fig. 1, well f, CM106 did not regain any of the Leu-Gly-hydrolyzing enzymes missing from CM89.

Utilization of peptides by peptidase mutants. The patterns of peptide utilization of the parent strain (CM17), a multiply peptidase-deficient strain (CM89), and the series of strains containing only one of the five peptidases missing in CM89 are presented in Table 5. Several conclusions can be drawn from these data. (i) Even the most peptidase-deficient strain we have obtained (CM89) still is able to use certain peptides. Gly-Leu serves as a leucine source, and both Met-Ala-Ser and Met-Ala-Met are methionine sources. This strain must still contain functional transport systems for both di- and oligopeptides, and it must also contain other peptidases. We have commented above on three of these activities: the *R_f* ~ 0.28 peptidase, the *R_f* ~ 0.47 activity, and peptidase P (an X-Pro-hydrolyzing enzyme). (ii) The overlapping

specificities of peptidases N, A, B, and D are strikingly demonstrated. Many of the leucine-containing dipeptides are hydrolyzed sufficiently rapidly by any one of these four enzymes to allow the peptide to be used as a leucine source. In agreement with the specificity patterns determined by activity staining following electrophoresis, the *pepD*⁺ *pepN* *pepA* *pepB* strain (CM107) differs from its *pepD* *pepN* *pepA* *pepB* parent only in the utilization of dipeptides. (iii) Clearly the peptidase activity stain is sensitive enough to detect activities that cannot function in vivo at a rate sufficient to allow growth on a particular peptide. Both the *R_f* ~ 0.28 and the *R_f* ~ 0.47 activities show weak activity toward Leu-Gly-Gly, for example, but mutants that still have these activities but lack peptidases N, A, and B fail to use this peptide as a leucine source. (iv) Peptides with N-terminal proline are clearly hydrolyzed by broad-specificity peptidases and do not require specific enzymes. Pro-Leu is a substrate for both peptidases A and D. X-Pro peptides, however, require specific peptidases (peptidase P or Q) for their hydrolysis. (v) It is conceivable that peptidases in addition to those we have observed are present in *E. coli* but cannot be observed because they have been inactivated by the electrophoresis or because the conditions of the activity stain are not optimal for their detection. However, our most deficient strain fails to grow on most of the leucine peptides tested, and the peptide utilization patterns of each of the "one-peptidase" transductants are consistent with the specificities of these

TABLE 5. Patterns of peptide utilization

Peptides	Utilization by strain:						
	CM17 (D,N,A,B,Q) ^a	CM89 (D,N,A,B,Q/—)	CM103 (D,A,B,Q/N)	CM104 (D,N,B,Q/A)	CM105 (D,A,N,Q/B)	CM107 (A,N,B,Q/D)	CM106 (D,A,N,B/Q)
As Leu source:							
Leu	+	+	+	+	+	+	+
Leu-Arg	+	—	+	+	—	+	—
Leu-Gly	+	—	+	+	+	+	—
Leu-Pro	+	—	—	—	—	—	+
Leu-Tyr	+	—	+	+	+	+	—
Ala-Leu	+	—	+	+	+	+	—
Arg-Leu	+	—	+	+	—	+	—
Asp-Leu	+	—	+	+	+	+	—
Gly-Leu	+	+	+	+	+	+	+
Pro-Leu	+	—	—	+	—	+	—
Trp-Leu	+	—	+	+	+	+	—
Leu-Ala-NH ₂	+	—	+	+	—	—	—
Leu-Gly-Gly	+	—	+	+	+	—	—
Leu-Leu-Leu	+	—	+	+	+	—	—
As Met source:							
Met	+	+					
Met-Ala-Ser	+	+					
Met-Ala-Met	+	+					

^a Parentheses indicate peptidases absent/peptidases present.

enzymes observed after gel electrophoresis. Therefore, if other peptidases are present but have escaped detection, they must be unable to hydrolyze many small leucine peptides.

DISCUSSION

The peptidases present in soluble protein extracts of *E. coli* K-12 are similar in both electrophoretic mobility and substrate specificity to those previously observed in *Salmonella*. Our results indicate that every activity observed by the gel electrophoresis activity stain procedure in *Salmonella* has a counterpart in *E. coli* with a similar (though not necessarily identical) electrophoretic mobility and a very similar substrate specificity profile. As discussed above, it seems likely that the gel electrophoresis activity stain procedure detects all of the peptidases capable of rapid hydrolysis of small leucine peptides. In two cases, the proposed homology between *E. coli* and *Salmonella* peptidases is supported by the similar map positions of mutations leading to loss of the peptidases. We have previously presented evidence (13) and have confirmed in this paper that the position of the *E. coli pepD* locus is near *proAB*. *Salmonella pepD* mutations are cotransducible with *proAB* (13). Latil et al. (6) have reported that *E. coli pepN* mutations are cotransducible with *pyrD*. We had previously observed cotransduction between *pepN* and *pyrD* in *Salmonella* (11).

The peptidase activities we have observed in *E. coli* can also be compared with activities from this organism previously studied by others. In three cases (peptidases A, N, and P), such comparison suggests definite correspondence between the activities we have observed and enzymes studied previously by other workers. We have argued elsewhere (13) that peptidase A is probably the aminopeptidase I purified from *E. coli* K-12 by Vogt (24). Aminopeptidase I is a high-molecular-weight, broad-specificity aminopeptidase that is characteristically heat resistant. This peptidase aggregates at low ionic strength, and it seems likely that its inability to enter the 7% acrylamide gel is the result of such aggregation (13). Peptidase N is the only enzyme with naphthylamidase activity present in either *Salmonella* or *E. coli*. It seems almost certain, therefore, that our peptidase N is the same enzyme as that purified from *E. coli* K-12 by Yang and Somerville (25) and from *E. coli* K-10 by Lazdunski and co-workers (7, 8). Yang and Somerville estimate that peptidase N comprises approximately 1% of the extractable protein of *E. coli* K-12. Although peptidase N is present in the soluble protein fraction after cell lysis and is not released from most strains by osmotic shock, it does seem to be associated in

some way with the cytoplasmic membrane (15). The correspondence of the activity we have called peptidase P with aminopeptidase P studied by Yaron and Berger (26) has been discussed above.

For several of the peptidase activities there is only suggestive evidence on which comparisons with previously studied enzymes can be made. Simmonds and co-workers (19) have reported similarities in electrophoretic mobility and substrate specificity between the *E. coli* K-12 peptidases studied by her group and the *Salmonella* peptidases. These results (19) suggest the following possible correspondences: (i) the $R_f \sim 0.28$ peptidase and aminopeptidase L, (ii) peptidase B and aminopeptidase AP, (iii) the $R_f \sim 0.47$ peptidase and tripeptidase TP, (iv) peptidase D and dipeptidase DP, and (v) peptidase N and oligopeptidase. These assignments now rest on only suggestive evidence, and further characterization of both the enzyme activities and the mutant strains will be required to test their validity. Two different dipeptidases have been purified from *E. coli* B (2, 16). It has been suggested (19) that dipeptidase DP from *E. coli* K-12 is probably analogous to the *E. coli* B dipeptidase studied by Brown (2), but different from that characterized by Patterson and co-workers (16).

Three reports of *E. coli* peptidase mutants have appeared previously. Kessel and Lubin (5) isolated a mutant of *E. coli* W that was unable to use Gly-Gly as a source of glycine. This strain was shown to contain reduced levels of a Gly-Gly-hydrolyzing peptidase. To our knowledge, no further genetic or biochemical characterization of this strain has been reported. If a Gly-Gly-specific dipeptidase is present in *E. coli* K-12 it could not be detected by the activity stain procedure we use because glycine is not a substrate for L-amino acid oxidase. *E. coli* mutants lacking peptidase N have been previously isolated using ANA as a chromogenic substrate to detect enzyme-deficient colonies (6). Other mutants have been isolated by Sussman and Gilvarg by screening for mutants sensitive to tryllysine. These mutants lack an enzyme capable of hydrolyzing tryllysine. Based on the substrate specificities and electrophoretic behavior reported by Simmonds and co-workers (19), this enzyme may be peptidase N.

The procedures reported in this paper for isolating *E. coli* peptidase mutants have several convenient features. First, the method involves a positive selection; no penicillin enrichment step is required. Such penicillin enrichment is required when peptidase mutants are isolated by screening for failure to utilize a peptide (13). Second, the selection is strong enough and the frequency of occurrence of peptidaseless mu-

tants among the various types of valine peptide-resistant clones is high enough that spontaneous mutants are easily isolated. It is particularly important to avoid repeated mutagenesis when several successive selections are required for obtaining multiply deficient mutants. It is also convenient to have positive selections for both loss (valine peptide resistance) and return (growth on peptides) of the peptidases.

Each type of peptidase mutant was isolated by selection for resistance to a particular valine peptide or, for *pepN* mutants, by screening for clones deficient in naphthylamidase activity. It is therefore possible that some of the mutants alter the substrate specificity of particular peptidases without totally inactivating these enzymes. We have observed, however, that mutation to resistance to a valine peptide corresponds in all the mutants tested with the loss of activity toward one or more leucine peptides. These results cannot rule out the possibility that some of our mutations may lead to specificity changes, but they provide no support for the idea.

The properties of the peptidase mutants show that one physiological function for these enzymes is hydrolysis of exogenously supplied peptides. The existence of specific and efficient uptake systems as well as a group of peptidases with broad and overlapping specificities suggests peptides may be important in nature as nutritional sources for these organisms. The mutants described here show much more limited capabilities for peptide utilization (Table 5) than their parents. Some peptides can still be used, however (Table 5), so the peptide uptake systems must still be present as well as other peptidases. It is likely that valine-containing peptides that are substrates for these remaining peptidases can be found and used to isolate mutants deficient in these enzymes. For example, our most deficient strain still contains aminopeptidase P. Peptides of the type Val-Pro-X would probably be rapidly hydrolyzed by this enzyme and could be used to isolate *pepP* mutants.

ACKNOWLEDGMENTS

We gratefully acknowledge the technical assistance of Carol Heiman and Judy Miller. Certain strains and helpful advice were provided by David Zipser.

This investigation was supported by Public Health Service research grant AI-1033 from the National Institute of Allergy and Infectious Diseases and Research Career Development Award GM-70321 from the National Institute of General Medical Sciences (C.G.M.). G.S. was an Undergraduate Research Participant (1972-1973).

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