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Genetics and Regulation of Peptidase N in *Escherichia coli* K-12

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Escherichia coli K-12 strains contain a cytoplasmic activity, peptidase N, capable of hydrolyzing alanine-*p*-nitroanilide. Mutations in the structural gene for the enzyme, *pepN*, were mapped, and the properties of mutant strains were examined. The *pepN* locus lay between *ompF* and *asnS* at approximately 20.8 min on the *E. coli* chromosome. Loss of peptidase N activity through mutation had no apparent effect on the growth rate or nutritional needs of the cell. Enzyme levels in wild-type strains were constant throughout the growth cycle and were constitutive in all of the growth media tested. Starvation for carbon, nitrogen, or phosphate also did not alter enzyme levels. Constitutive expression of peptidase N is consistent with the idea that the enzyme plays a significant role in the degradation of intracellularly generated peptides.

In the bacterial cell, peptides arise primarily from the processing of leader sequences of exported proteins (5) and the turnover of denatured or abnormal proteins (3). The fact that these peptides do not normally accumulate in gram-negative bacteria can be attributed to the presence of numerous peptidase activities (14).

One such activity, peptidase N, has been identified in both *Escherichia coli* and *Salmonella typhimurium* (7, 9, 12, 16, 17) as an aminopeptidase that hydrolyzes alanine-containing pseudopeptide chromogenic substrates. The enzyme has no endoproteolytic activity but does cleave small hydrophobic peptides (12). Loss of peptidase N through mutation in *S. typhimurium* contributes to a decrease in the overall rate of protein degradation during starvation (26). Strains made multiply deficient in peptidase activities by mutation also accumulate small peptides (27). It has been difficult, however, to assign a specific metabolic role to each of the many known peptidase activities.

As a further step toward understanding the role of peptidase N in protein degradation and other physiological processing events, we isolated and characterized mutant strains of *E. coli* lacking peptidase N and mapped the corresponding *pepN* locus. The regulation of peptidase N activity was also examined.

MATERIALS AND METHODS

Bacterial strains. All bacterial strains are derivatives of *E. coli* K-12 and are described in Table 1.

Media and growth conditions. Minimal medium was composed of M63 salts (19) and supplemented with 0.4% of the specified carbon source. Media were supplemented with L-amino acids at 40 μg/ml as required for amino acid auxotrophs. Luria broth media (19) containing 0.4% glucose or M63-glycerol enriched with 0.5% peptone (Difco Laboratories) were used as enriched media. Solid media contained 1.5% agar (Difco). Streptomycin (100 μg/ml), ampicillin (30 μg/ml), and tetracycline (25 μg/ml) were used as counterselective agents in agar plates. For phosphate starvation, phosphate-free M63 medium buffered with 100 mM Tris was used. MacConkey agar (Difco) was used to screen for lactose utilization. All liquid cultures were grown in a rotary shaker at 200 rpm.

Mutagenesis. Diethyl sulfate (DES; Sigma Chemical Co.), an alkylating agent known to cause base substitutions, was used to generate localized defects in the *pepN* gene (22). Infection and chromosomal insertion of the Mu *cts* (*dlac* Ap) phage produced mutant strains totally lacking enzyme activity. Preparation of Mu phage lysates and isolation of lysogens were carried out as described by Casadaban and Cohen (2).

Peptidase mutant isolation. Peptidase N-deficient mutants were identified by a modification of the staining procedure described by Miller (16), with a soft agar overlay containing 1.5 mM alanyl-β-naphthylamide (Sigma) and 2.5 mg of *o*-amino azotoluene, diazonium salt (Sigma). Nonstaining colonies were picked and restreaked twice before being characterized. All mutant strains were also nonstaining when 3 μl of 50 mM alanine-*p*-nitroanilide (Ala-pNA) was spotted on individual colonies, whereas wild-type colonies turned yellow. All peptidase N mutant strains maintained parental nutritional requirements.

Enzyme assays. Enzyme activities were measured in soluble extracts of cells prepared by sonication for 3 min in an ice bath, followed by centrifugation at 87,000 × *g* for 60 min in a Beckman Ti60 rotor. Peptidase N activity was measured by following the hydrolysis of 1 mM Ala-pNA (Chemalog) at 410 nm (12). β-Galactosidase was measured by the hydrolysis of *o*-nitrophenyl-β-D-galactoside at 420 nm (4). Alkaline phos-

TABLE 1. *E. coli* strains

Strain	Genotype	Source or manipulation/ parent
Other workers		
MX419	*thi relA lac nal rpsL tsx* λ^- *supD*	B. Bachmann
G19	*putA purE gltA his lac strA*	B. Bachmann
Hfr3000	(HfrH) *thi relA spoT* λ^-	B. Bachmann
KL19	Wild-type	B. Bachmann
DG30	*proA aspC his ilvE argE thi tyrB hsd hpp sbcB lac gal xyl mtl rps str supE recB recC* λ^-	B. Bachmann
KLF6/KL181	(F′ 106) *thi pyrD his trp recA galK mtl xyl malA strA* λ^r λ^-	B. Bachmann
KLF25/KL181	(F′ 125), same as KL181 above	J. Ingraham
AB1157	*thi leu lac gal ara xyl mtl pro his arg str tsx sup* λ^- λ^s	A. J. Clark
W4680	Δ*lacZ str gal*	I. Zabin
DC304	*zcb-222*::Tn*10 trp his pyrD thi gal mal xyl mtl rpsL*	D. Clark
AM102	Mu cts (d*lac* Ap)::*sulA thr leu thi argE his proA lon lacY galK mtl xyl ara strA tsx* λ^- *supE pyrD*	A. McPartland
NO1801	*thi str galK relA asnS*	M. Nomura
MH450	*ompF*::Tn*5* Δ(*lac*)*U169 rpsL relA thiA flbB*	T. Silhavy
This study		
MRV1	*thi leu lac gal ara xyl mtl pro his arg str supE tsx* λ^- λ^s *pepN301*	DES/AB1157
MRV6	*thi relA lacZ nalA strA tsx* λ^- *supD pepN306*	DES/MX419
MRV8	*pepN308*	DES/KL19
MRV28	*lacZ str gal pepN*::Mu cts (d*lac* Ap)	Mu infected/W4680
MRV30	*lacZ str gal pepN*::Mu cts (d*lac* Ap)	Mu infected/W4680
MRV41	Temp^r Mu lysogen in MRV28	Spontaneous/MRV28
MTM22	*pyrD zcb*::Tn*10 pepN308*	Transduction/MRV8
MTM26	*putA lac str*	Transduction/G19
MTM31	*proA his ilvE argE thi tyrB hsd hpp lac gal xyl mtl rps str supE recB recC sbcB* λ^- *pepN308*	Transduction/DG30
MTM32	*thi str galK relA asnS pryD zcb*::Tn*10*	Transduction/NO1801
MTM33	*thi str galK relA asnS pepN308*	Transduction/MTM32

phatase was measured by following the hydrolysis of *o*-nitrophenylphosphate at 410 nm (12). Reagents for these assays were obtained from Sigma. Protein was measured by the method of Lowry (11), with bovine serum albumin used as a standard.

Episomal transfer. Strain KLF6/KL181 contains an episome (F′106) that carries genes in the region of 20 to 22 min on the *E. coli* chromosome (10, 22). Transfer of this episome, by F-duction, was used to verify the map position of *pepN* and to test the dominance of mutations in this region of the chromosome. Donor strain KLF6/KL181 was grown statically overnight in Penassay broth (Difco). Recipients were grown with shaking overnight in the same medium. Donor cells (1 ml) were transferred to 1 ml of fresh medium and grown for 1 h, at which time 10^9 recipient cells were added. After a 1-h incubation at 37°C, the mixed culture was diluted in saline and spread on minimal medium containing streptomycin and lacking uracil; the resulting *pyrD*$^+$ or *aspC*$^+$ recombinants were screened for other phenotypic properties.

P1 transductions. Transducing lysates of P1 *vir* were prepared as described by Miller (19). Phage infections were done at a multiplicity of 0.1 in Luria broth containing 5 mM $CaCl_2$ and 10 mM $MgSO_4$. After incubation at 37°C for 20 min, the infected cells were collected by centrifugation, washed twice in saline, and spread on selective medium. The spontaneous reversion rate of each mutant phenotype was insignificant compared with the frequency of transduction of the wild-type allele. For crosses in which the selection was for kanamycin resistance (*ompF*::Tn*5* donor), the transduced cells were diluted into fresh liquid medium and grown overnight for phenotypic expression before being plated on kanamycin-supplemented medium.

Mutant phenotype screening. Loss of peptidase N activity was determined by staining individual colonies with 3 μl of 50 mM Ala-pNA. Strains containing a *pyrD* lesion required uracil (40 μg/ml) for growth. The *asnS* defect is a temperature-sensitive mutation that allows cell growth at 30 but not at 42°C. The *aroA* mutation results in auxotrophic requirements for tryptophan, tyrosine, and phenylalanine. The *putA* defect prevents the utilization of proline when provided as the sole nitrogen source in minimal medium (21). The presence of the Tn*10* element (strain DC304) allowed growth on Luria broth containing tetracycline. Resistance to ampicillin was evidence for the presence of the Mu (d*lac* Ap) phage lysogen. The *ompF*::Tn*5* genotype was verified by examination of the outer membrane composition of strain MH450 to confirm the absence of the *ompF* protein. *OmpF*::Tn*5* recombinants were scored by kanamycin resistance.

RESULTS

Isolation of peptidase N mutant strains. Peptidase N-deficient point mutants resulted from treatment of *E. coli* K-12 with the chemical mutagen DES. Mutants were also obtained by

lysogenization with the Mu cts (d*lac* Ap) phage of Casadaban and Cohen (2) to define the consequences of complete loss of function and to facilitate studies of gene regulation. Mutant colonies were identified in qualitative plate staining assays by their inability to hydrolyze chromogenic substrates. Hydrolysis products of alanyl-β-napthylamide react in a dye-coupled reaction to form a red precipitate within wild-type colonies. Alternatively, a drop of 50 mM Ala-pNA caused peptidase N-containing colonies to turn yellow. A total of 37 nonstaining, peptidase N-deficient mutants were isolated from seven parental strains. At least 20 of these mutants resulted from independent mutational events. Wild-type and purified mutant strains were grown in liquid culture and lysed by sonication, and the specific activity of peptidase N in the soluble fractions was measured with Ala-pNA used as a substrate. The spectrum of peptidase-deficient mutants obtained ranged from those having 10% of wild-type activity to those with undetectable activity. Mutant strains used for genetic mapping and physiology studies had no more than 3% of wild-type activity (Table 2). All mutant strains lacking Ala-pNA hydrolytic activity also lost the ability to hydrolyze the other chromogenic substrates recognized by purified peptidase N (12) (data not shown).

Genetic mapping of the *pepN* locus. Two different *pepN* mutations, *pepN301* and *pepN308*, were chosen for genetic mapping studies. These DES-induced lesions represented two mutational classes. Strains carrying *pepN301* showed 2 to 3% of the wild-type peptidase N levels, whereas *pepN308* mutants had no detectable enzyme activity. Both mutations were localized to the 20.5 to 22.5-min region of the *E. coli* chromosome by Hfr mating experiments and phage P1 cotransduction studies. The phage crosses demonstrated linkage of *pepN* to the *pyrD* (63%) and *aroA* (31%) loci, and no linkage to *putA* (0%). These observations were in agreement with those of Latil et al. (7), who reported cotransduction of the *pepN* locus with *pyrD* and *fabA* (Fig. 1).

TABLE 2. Peptidase N activity in wild-type and *pepN* mutant strains[a]

Strain (relevant genotype)	Peptidase N activity (U/mg of protein)
Wild type	
Hfr3000	0.20
AB1157	0.16
A4680	0.16
KLF25/KL181 (F′ 125)	0.45
KLF6/KL181 (F′ 106)	0.77
Mutant	
MRV1 (*pepN301*)	0.004
MRV6 (*pepN306*)	0.001
MRV8 (*pepN308*)	<0.0004
MRV28 (*pepN328*::Mu d*lac*)	<0.0003
MRV30 (*pepN330*::Mu d*lac*)	<0.0002
KLF6/MTM22 (*pepN308*)	0.26

[a] Strains were grown to late exponential phase, harvested, and sonicated. Crude extracts were centrifuged (20,000 × *g* for 15 min), and kinetic assays were performed on the clarified extracts as described in the text.

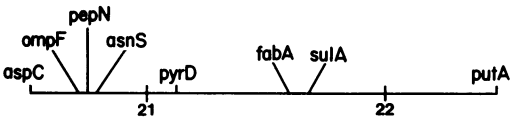
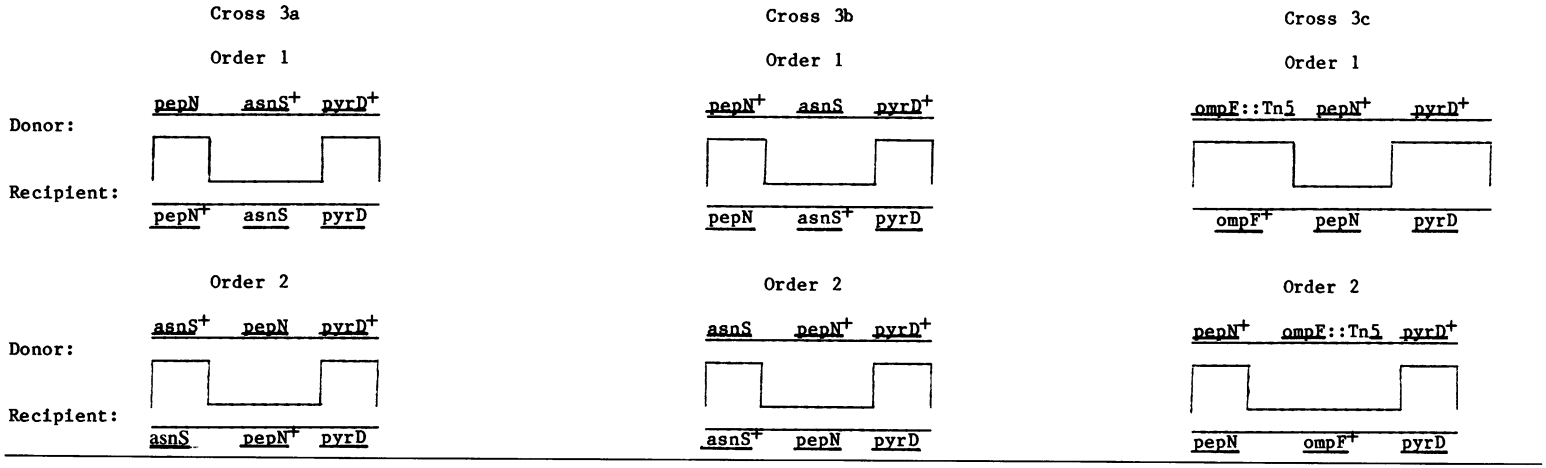


FIG. 1. Position of *pepN* on the genetic map of *E. coli*. Adapted and expanded from the genetic map of Bachmann and Low (1).

More precise localization of *pepN* was accomplished by three-factor crosses. Table 3 shows the results of a genetic cross designed to order *pepN* with respect to *pyrD* and *asnS*. A donor P1 *vir* lysate grown on strain MRV8 (*pyrD*$^+$ *asnS*$^+$ *pepN308*) was used to transduce a *pyrD asnS pepN*$^+$ recipient with selection for *pyrD*$^+$. The pattern of marker segregation was consistent with the gene order *pepN asnS pyrD*, i.e., *pyrD*$^+$ *asnS pepN* is a rare class. A similar conclusion was derived from the reciprocal cross (Table 3, experiment B). In this experiment a donor lysate grown on a *pyrD*$^+$ *asnS pepN*$^+$ strain was used to transduce a *pyrD asnS*$^+$ *pepN308* recipient. The frequencies of unselected markers among the *pyrD*$^+$ transductants indicated that a quadruple crossover event yields *pyrD*$^+$ *asnS*$^+$ *pepN*$^+$ transductants, the rare class. The positioning of *pepN* to the left of *pyrD* is supported by the results of the three-factor cross shown in experiment 3D. Segregation of the unselected markers *pepN* and *sulA* among *pyrD*$^+$ recombinants was determined for a cross between strain MRV1 (*pepN pyrD*$^+$ *sulA*$^+$) and a *pepN*$^+$ *pyrD sulA*::Mu (d*lac*) recipient. The relatively common occurrence of all four possible recombinant classes indicated that *pepN* lies on the *sulA*-distal side of *pyrD*, in agreement with the gene order established by the *pepN-asnS-pyrD* crosses.

In the region of the chromosome between 20 and 21 min, the established gene order is *aspC ompF asnS pyrD* (Fig. 1). The results of a three-factor cross employing an *ompF*::Tn*5* *pepN*$^+$ *pyrD*$^+$ donor and an *ompF*$^+$ *pepN308 pyrD* recipient indicated close linkage of *pepN* and *ompF*, and the pattern of marker segregation among *pyrD*$^+$ recombinants was consistent with the order *ompF pepN asnS* (Table 3, experiment

TABLE 3. Three-factor transductional mapping of *pepN*[a]



Expt	Donor strain (relevant genotype)	Recipient strain (relevant genotype)	Selected marker	Unselected classes	Frequency	
					No.	%
A	MRV1 (*pepN308 asnS*$^+$ *pyrD*$^+$)	MTM32 (*pepN*$^+$ *asnS pyrD*)	*pyrD*$^+$	*pepN asnS*$^+$	208	51
				pepN$^+$ *asnS*$^+$	68	17
				pepN asnS	6	1.5
				pepN$^+$ *asnS*	127	31
B	NO1801 (*pepN*$^+$ *asnS pyrD*$^+$)	MTM22 (*pepN308 asnS*$^+$ *pyrD*)	*pyrD*$^+$	*pepN asnS*$^+$	100	29
				pepN$^+$ *asnS*$^+$	5	1.4
				pepN asnS	62	18
				pepN$^+$ *asnS*	182	52
C	MH450 (*ompF*::Tn*5 pepN*$^+$ *pyrD*$^+$)	MTM22 (*ompF*$^+$ *pepN308 pyrD*)	*pyrD*$^+$	*ompF*::Tn*5 pepN*$^+$	199	45
				ompF$^+$ *pepN*$^+$	52	12
				ompF::Tn*5 pepN*	3	0.7
				ompF$^+$ *pepN*	187	42
D	MRV1 (*pyrD*$^+$ *pepN301 sulA*$^+$)	AM102 [*pyrD pepN*$^+$ *sulA*::Mu (d*lac* Ap)]	*pyrD*$^+$	*pepN*$^+$ *sulA*::Mu (d*lac*)	41	9
				pepN$^+$ *sulA*$^+$	85	18
				pepN sulA::Mu (d*lac*)	57	12
				pepN sulA$^+$	280	60

[a] *pyrD*$^+$ transductants detected by plating on medium lacking uracil were purified by colony transfer to the same medium with sterile toothpicks. Ura$^+$ colonies were tested for the *asnS* lesion by transfer to each of two fresh plates for incubation at 30 and 42°C, respectively. Temperature sensitivity was taken as indication of the *asnS* genotype. The presence of the *ompF*::Tn*5* insertion was monitored by kanamycin resistance (40 μg/ml), and the *sulA*::Mu (d*lac* Ap) insertion was detected by ampicillin resistance (25 μg/ml). Purified colonies were tested for *pepN* as described in the text.

C). Direct selection for kanamycin resistance (*ompF*::Tn*5*) verified the high linkage between *pepN* and *ompF* seen with the *pyrD*$^+$ selection (data not shown).

pepN301 and *pepN308* may be structural gene mutations, since full activity was restored when the F'106 episome (20 to 22 min) was introduced into these mutant strains. In addition, a gene dosage effect was seen in wild-type strains carrying the F'125 or F'106 episomes (Table 2).

Regulation of peptidase N. The possibility of regulatory interaction between *pepN* and the neighboring *ompF* gene was explored. We first examined the outer membrane composition of strain MRV1 (*pepN301*) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. No alteration in *ompF* protein levels were detected compared with wild-type strains. Conversely, peptidase N enzyme levels were measured in *ompF* mutant strains [both a point mutant and a Mu (d*lac*) insertion mutant] and found to be equal to wild-type levels, indicating no coordination of expression of *pepN* and *ompF*.

To study the physiological regulation of peptidase N activity, we measured enzyme levels in cultures grown under different conditions, including starvation for phosphate, carbon, and nitrogen sources. An exponentially growing culture of strain Hfr3000 was transferred to phosphate-free (Tris-M63) medium, and the levels of alkaline phosphatase and peptidase N were monitored. After 3 h of phosphate limitation, alkaline phosphatase activity had increased at least 50-fold, but no change was observed in peptidase N enzyme levels (Fig. 2). Since earlier reports had suggested that *pepN* was induced by phosphate limitation in *E. coli* (20), we also looked for phosphate regulation of a Mu (d*lac*) insertion in *pepN* (strain MRV41) and found none. In agreement with earlier reports, we found that mutations in the regulatory gene *phoR* rendered alkaline phosphatase expression constitutive but had no effect on peptidase N levels. Carbon or nitrogen starvation also did not alter enzyme levels (Table 4). The specific activity of the enzyme remained constant during all phases of growth in a liquid culture. Growth on M63 salts supplemented with glucose, glycerol, lactose, maltose, or galactose resulted in no significant variation in peptidase N levels. β-Galactosidase activity of several *pepN*::Mu (d*lac*) insertion mutant strains was assayed as an alternative measure of *pepN* transcription (2). β-Galactosidase levels were constitutive, unchanged by culture growth stage and unaffected by medium composition in a manner identical to changes in peptidase N levels in wild-type strains (Table 4). Although the β-galactosidase levels in the *pepN*::Mu (d*lac*) strain were constant, they were only 2% of the maximal enzyme levels achieved by induction of an intact *lac* operon (Table 4, footnote *a*).

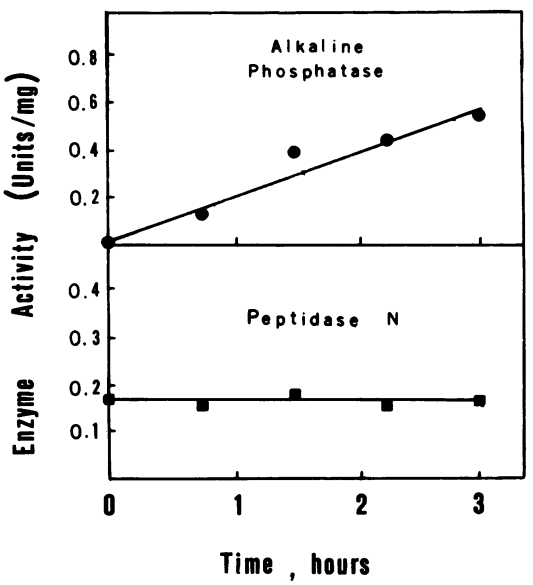


FIG. 2. The effect of phosphate limitation on peptidase N levels. Strain Hfr3000 was grown on M63-glycerol to exponential phase. Cells were washed twice and suspended in phosphate-free medium (see the text). Samples were removed at 45-min intervals and sonicated, and the soluble fractions were assayed for protein content and enzyme activities. Activities are expressed as micromoles of product per minute per milligram of protein.

TABLE 4. Peptidase N levels after starvation for carbon and nitrogen sources[a]

Medium	Sp act (U/mg of protein)[b]	
	Peptidase N (Hfr3000)	β-Galactosidase (MRV41)
Control (M63-glycerol)	0.21	151
M63, no carbon source	0.17	102
M63-glycerol, no nitrogen source	0.16	—
Minimal salts, no carbon or nitrogen source	0.19	—

[a] Exponentially growing strains Hfr3000 and MRV41 were centrifuged, washed, and suspended in the indicated media. After 4 h of incubation, cells were harvested and enzyme activity was assayed on sonicated extracts as described in the text. β-Galactosidase in strain MRV41 [*pepN*::Mu (d*lac*)] is under *pepN* promoter transcriptional control. As a reference value, β-galactosidase was assayed in strain Hfr3000 (intact *lac* operon) after 2 h of growth on M63-glycerol containing 0.5 mM isopropyl-β-D-thiogalactopyranoside. The specific activity of the enzyme was 12,350 U/mg of total cell protein under these conditions.

[b] —, None detected.

DISCUSSION

As part of a study of the role of peptidase N in cellular metabolism in *E. coli*, we mapped the *pepN* gene and looked for possible genetic or physiological regulation. Genetic mapping of peptidase N-deficient strains by three-point P1 transduction crosses showed that *pepN* defects are located at 20.8 min on the *E. coli* chromosome, in agreement with earlier approximations in *E. coli* (7). Our results suggested a gene order of *ompF pepN asnS pyrD putA* (Fig. 1). Placement of *pepN* on the side of *pyrD* distal to *putA* is also likely in *S. typhimurium* (13). Gene dosage effects and the dominance of the wild-type *pepN* allele carried on an episome suggested that the *pepN* lesions studied are structural gene defects. We detected no evidence for regulatory gene involvement in the expression of *pepN*.

Previous reports (8, 20) suggested that *pepN* is, in part, regulated by external phosphate levels. Phosphate limitation normally results in the derepression of several genes involved in phosphate uptake and utilization, including *phoA* (alkaline phosphatase) (24). These protein products are constitutively synthesized in strains with mutations in the regulatory gene *phoR*. Our observations show that peptidase N levels are constitutive and unaltered by physiological or genetic manipulations that result in greatly elevated levels of alkaline phosphatase. Therefore, in the strains studied, *pepN* is not coregulated with the genes comprising the cellular response to phosphate limitation.

At one time it was believed that the primary function of peptidases was the cleavage of exogenous peptides for nutrition. More recently, Miller and coworkers have shown that several peptidases, including peptidase N, appear to function in both a catabolic and intracellular protein turnover role (15, 18, 26, 27). Little is known about the regulation of peptide transport systems and their extent of interaction with cellular peptidase activities (6, 23). Thus, it is difficult to separate the possible catabolic and protein turnover roles of peptidases. Our investigation of the physiological regulation of peptidase N indicated that enzyme levels are unaffected by carbon source or growth stage in liquid culture. No significant increase was seen in peptidase N levels during growth on enriched media (containing external peptides). In peptidase N mutant strains containing Mu (d*lac*) phage inserts under transcriptional control of the *pepN* promoter, β-galactosidase activity reflected the same lack of response to media variation as did the authentic gene product. However, the specific activity of β-galactosidase in the Mu insert indicates a much lower transcription rate from the *pepN* promoter than from a normal *lac* promoter. This, and our previous measurement of peptidase N in crude cell extracts (12), suggest that an earlier estimate of peptidase N protein as 1% of the soluble protein in *E. coli* was excessive (25). Starvation for carbon or nitrogen sources also had no effect on peptidase N or β-galactosidase under *pepN* promoter control. Since overall protein turnover rates are elevated by starvation (3), peptidase N action is not likely to be the rate-limiting step in the process. This is also consistent with the small-substrate-size limitations of peptidase N (12) and the idea that control of protein degradation is likely to be exerted at some early, perhaps energy-dependent, endoproteolytic reaction (3).

The observation that peptidase N expression is constitutive and does not respond significantly to changes in the external environment of the cell (27) is consistent with the idea that peptidase N plays a significant role in the hydrolysis of intracellularly generated peptides in the final stages of protein degradation in the cell.

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