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Aminopeptidases A, B, and N and Dipeptidase D Are the Four Cysteinylglycinases of *Escherichia coli* K-12

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Aminopeptidases A, B, and N and dipeptidase D, with broad substrate specificity, are the four cysteinylglycinases of *Escherichia coli* K-12, and there is no peptidase specific for the cleavage of cysteinylglycine.

Glutathione is a tripeptide with the structure L-γ-glutamyl-L-cysteinylglycine. *Escherichia coli* K-12 synthesizes glutathione and, during the exponential and early stationary phases, excretes into the medium some glutathione (9, 16), which is subsequently utilized during the stationary phase (16). γ-Glutamyltranspeptidase existing in the periplasm (14) cleaves the γ-glutamyl linkage of glutathione to generate glutamate and cysteinylglycine, and cysteinylglycine is taken up into the cytoplasm and utilized as both cysteine and glycine sources (12). In a previous paper, we proposed that this is the cysteine salvage pathway in *E. coli* K-12 (13). Thus, the next step was to identify which peptidase of *E. coli* K-12 is responsible for the cleavage of the peptide bond of cysteinylglycine between cysteine and glycine. Since McCorquodale's description of cysteinylglycinase activity in *E. coli* B (3), there have been no reports on cysteinylglycinase of *E. coli*. Miller and his coworkers performed an extensive study on peptidases of *E. coli* K-12 and *Salmonella enterica* serovar Typhimurium using peptidase-deficient strains and elucidated their physiological roles (reviews in references 4 and 5). They reported that one of the physiological roles of cytoplasmic dipeptidases and aminopeptidases is the hydrolysis of peptides supplied exogenously, which allows the peptides to be used as amino acid sources. However, although they investigated the substrate specificity of peptidases, they did not investigate whether these peptidases are able to cleave the peptide bond of cysteinylglycine or if there is another cysteinylglycinase different from the peptidases they described (7). In this study, the peptidases of *E. coli* K-12 responsible for the cleavage of cysteinylglycine were identified.

L-Cysteinylglycine and L-leucylglycine were purchased from Sigma Chemical Co. All strains used were *E. coli* K-12 derivatives and are listed in Table 1. Δ(*pro-lac*) deletes the *pepD* gene (7). All *pep* mutants were grown in Luria-Bertani broth (8) supplemented with 0.05 mM thymine and 0.03 mM thiamine at 37°C. As a minimal medium, M9 glucose medium (8) supplemented with 0.05 mM leucine, 0.3 mM methionine, 0.3 mM proline, 0.05 mM thymine, and 0.03 mM thiamine was used. When necessary, antibiotics and peptides were added. KES and SH strains were constructed by P1 *vir*-mediated transduction and Hfr mating (Table 1) as described previously (16).

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E. coli K-12 lacks valine-resistant acetohydroxy acid synthase and cannot grow on a minimal medium containing valine unless isoleucine is added (valine sensitivity of *E. coli* K-12) (17). A *pepABDN* mutant, such as strain CM86, is valylvaline resistant because only peptidases A, B, D, and N of *E. coli* K-12 can cleave the peptide bond of this dipeptide to liberate valine (7); in addition, reversion of any one of these peptidase genes makes the strain sensitive to valylvaline (at 0.25 mM). Therefore, when the *pepA*$^+$, *pepB*$^+$, *pepD*$^+$, or *pepN*$^+$ allele was introduced into a *pepABDN* strain by transduction or Hfr mating, tetracycline-resistant transductants and transconjugants were screened for valylvaline resistance and valylvaline-sensitive transductants and transconjugants were stored as Pep$^+$ strains. Cell extracts of these Pep$^+$ strains were subjected to native polyacrylamide gel electrophoresis (15), followed by peptidase activity staining using L-leucylglycine as a substrate (6). The peptidase bands formed were compared with those of the control strain to confirm that the Pep$^+$ phenotype was derived from the desired *pep*$^+$ gene introduced into the strain. The assay solution for cysteinylglycinase activity was comprised of 0.5 mM cysteinylglycine, 50 mM Tris-HCl (pH 7.5), and 1 mM $MnSO_4$, in a final volume of 0.1 ml. The reaction was carried out at 37°C and was terminated by the addition of 0.9 ml of 0.5 M potassium citrate buffer (pH 2.2). The amount of glycine released was measured with a high-performance liquid chromatograph equipped with a Shim-pack Amino-Na column and a fluorescence detector (model LC-9A; Shimadzu, Kyoto, Japan) with *o*-phthalaldehyde as the detection reagent. One unit of enzyme was defined as the amount of enzyme that released 1 μmol of glycine per min. Protein concentrations were measured by the method of Lowry et al. (2), with bovine serum albumin as a standard.

Aminopeptidases A, B, and N and dipeptidase D are known as peptidases with broad substrate specificity (7). Strain CM86, which has defects in all of these peptidases, showed no detectable cysteinylglycinase activity (Table 2). Strains that recovered one of these four peptidases were constructed, and their cysteinylglycinase activities were measured. All four strains recovered cysteinylglycinase activity (Table 2).

Cysteine auxotrophy was introduced into these strains, and utilization of cysteinylglycine as a cysteine source was tested (Table 3). Strain SH1420 could not grow on the minimal medium supplemented with cysteinylglycine as a cysteine source, while SH1429, SH1423, SH1424, and SH1426, which recovered peptidases A, B, N, and D, respectively, grew on the same plate.

TABLE 1. *E. coli* K-12 strains used in this study

Strain	Genotype	Source and/or reference
CAG12094	F⁻ *zcb-3059*::Tn*10*	C. A. Gross (10)
CAG12182	F⁻ *cysC3152*::Tn*10kan*	C. A. Gross (10)
CAG18481	F⁻ *zff-208*::Tn*10*	C. A. Gross (10)
CM86	F⁻ *pepA11 pepB1 pepN102* Δ(*pro-lac*) *leu-9 met thyA*	C. G. Miller (7)
CS101	Hfr PO2A *pyrE41 metB1 tonA22 relA1* T_2^r	C. A. Gross
GR401	F⁻ *araD139* Δ(*argF-lac*)*169 flbB5301* Δ(*his-gnd*) *cycA30*::Tn*10*	B. J. Bachmann
JW353	F⁻ *thr-1 leuB6 thyA6 met-89 thi-1 deoC1 lacY1 rpsL67 tonA21 supE44 zae-502*::Tn*10*	C. A. Gross
KES2	F⁻ *pepA11 pepB1 pepN102* Δ(*pro-lac*) *leu-9 met thyA rpsL*	Spontaneous Str^r of CM86 (this work)
KES8	Hfr PO2A *pyrE41 metB1 tonA22 relA1* T_2^r *zae-502*::Tn*10*	CS101 × P1(JW353) (this work)
KES11	F⁻ *pepA11 pepN102* Δ(*pro-lac*) *leu-9 met thyA zff-208*::Tn*10*	CM86 × P1(CAG18481) (this work)
KES13	F⁻ *pepA11 pepB1* Δ(*pro-lac*) *leu-9 met thyA zcb-3059*::Tn*10*	CM86 × P1(CAG12094) (this work)
KES17	F⁻ *pepA11 pepB1 pepN102 leu-9 met thyA rpsL zae-502*::Tn*10*	KES2 × KES8, Hfr mating (this work)
KES22	F⁻ *pepB1 pepN102* Δ(*pro-lac*) *leu-9 met thyA rpsL cycA30*::Tn*10*	KES2 × P1(GR401) (this work)
MG1655	Wild type	C. A. Gross
SH1420	CM86 but *cysC3152*::Tn*10kan*	CM86 × P1(CAG12182) (this work)
SH1423	KES11 but *cysC3152*::Tn*10kan*	KES11 × P1(CAG12182) (this work)
SH1424	KES13 but *cysC3152*::Tn*10kan*	KES13 × P1(CAG12182) (this work)
SH1426	KES17 but *cysC3152*::Tn*10kan*	KES17 × P1(CAG12182) (this work)
SH1429	KES22 but *cysC3152*::Tn*10kan*	KES22 × P1(CAG12182) (this work)

These results indicate that there is no peptidase specific for the cleavage of cysteinylglycine, but that any one of aminopeptidase A, B, or N or dipeptidase D is sufficient for *E. coli* to utilize cysteinylglycine as a cysteine source.

Using *S. enterica* serovar Typhimurium strain TA100, Glatt et al. found that glutathione in the presence of rat kidney homogenate was Ames test positive (1). Stark et al. showed that cysteinylglycine generated through the cleavage of glutathione by γ-glutamyltranspeptidase is subjected to auto-oxidation, with the production of free radicals that leads to hydrogen peroxide, the ultimate mutagen (11). Since strain CM86 was found to have no detectable cysteinylglycinase activity, the question of whether CM86 is more mutagenic than the control strain arose. The frequency of appearance of streptomycin-resistant mutants on the medium containing cysteinylglycine did not differ between strain CM86 and the control strain (data not shown). Although these *pep* mutations in the *S. enterica* serovar Typhimurium TA100 and TA102 backgrounds should be investigated, in our strain background, a deficiency of cysteinylglycinase had no effect on the mutagenicity of cysteinylglycine.

TABLE 2. Comparison of cysteinylglycinase activities of cell extracts

Strain	Relevant genotype	Sp act (mU/mg)
MG1655	*pepA*⁺*B*⁺*D*⁺*N*⁺	369
CM86	*pepABDN*	ND[a]
KES22	*pepA*⁺*BDN*	91
KES11	*pepAB*⁺*DN*	180
KES17	*pepABD*⁺*N*	60
KES13	*pepABDN*⁺	16

[a] ND, not detectable.

TABLE 3. Utilization of cysteinylglycine by peptidase-deficient strains

Strain	Relevant genotype	Growth on M9 minimal medium plate		
		Alone	+ Cys-Gly[a]	+ Cyn[b]
CM86	*cysC*⁺ *pepABDN*	+	+	+
SH1420	*cysC pepABDN*	−	−	+
SH1429	*cysC pepA*⁺*BDN*	−	+	+
SH1423	*cysC pepAB*⁺*DN*	−	+	+
SH1426	*cysC pepABD*⁺*N*	−	+	+
SH1424	*cysC pepABDN*⁺	−	+	+

[a] L-Cysteinylglycine was added to a final concentration of 0.6 mM.
[b] L-Cystine was added to a final concentration of 0.3 mM because strain CM86 is sensitive to L-cysteine.

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