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Isolation and Genetic Mapping of *Escherichia coli* Aminopeptidase Mutants

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Summary. Many mutant strains devoid of aminopeptidase activity have been isolated in *Escherichia coli*. All of them produce cross-reacting material when tested against specific antiaminopeptidase antibody. The map position of the locus specifying this enzyme has been determined by three conjugations and two P_1 mediated transduction experiments. By analogy with *Salmonella typhimurium* this locus has been called *pepN* (Miller, 1975). Mutations in *pepN* are jointly transduced with *fabA* and *pyrD* at high frequency. These data and conjugation results suggest a location between 20.5 and 22.5 minutes on *E. coli* genetic map.

Introduction

We have recently reported the presence of an aminopeptidase located near the cell surface in *Escherichia coli* (Lazdunski et al., 1975a). Although this enzyme is constitutively produced, the differential rate of synthesis is increased about 4-fold simultaneously with derepression of alkaline phosphatase synthesis upon phosphate starvation (Lazdunski et al., 1975a). None of the mutations involved in the control of alkaline phosphatase synthesis has any effect on aminopeptidase synthesis (Lazdunski et al., 1975b; Murgier et al., 1976a).

Since aminopeptidase shows a constitutive synthesis the question arises to know if this enzyme is dispensible for growth or not. The isolation of mutant strains which lack this peptidase activity might help to answer this question. Furthermore, these mutants might be used to investigate the physiological role of this enzyme.

In this study, we report the isolation and mapping of such a mutant. We have also looked for cross-reacting material (CRM) in various aminopeptidase negative mutants to characterize the type of mutations.

Materials and Methods

Materials

Ethyl methane sulfonate and Nitrosoguanidine were obtained from Koch-Light Laboratories. L-alanine-β-naphthylamide, p-nitrophenylphosphate, L-alanine-p-nitroanilide and Fast Garnet GBC were from Sigma Chemical Co.

Bacterial Strains

The strains carrying multiple markers used in this work are described in Table 1. All of them are *E. coli* K12 derivatives.

Media and Growth Conditions

Media and growth conditions used in physiological experiments have been already described (Lazdunski et al., 1975a). The media used in genetic experiments were: Minimal medium (Davis et al., 1950), Lennox medium (Lennox, 1955) and Nutrient Broth (Davis

Table 1. Bacterial strains

Strain	Sex	Genotype	Origin
303	Hfr P4x	met thi $(\lambda)^+$	E. Wollman
304	F^-	arg gal trp his lac thr leu str^r thi mal xyl mtl	Institut Pasteur W. Hayes
322	Hfr H	thi	
923	Hfr KL99	thi-1 rel-1 λ^- lac-42	B. Bachmann
927	Hfr KL983	xyl-7 lac Y-1 or lac Z-4 mglp-1 thi	B. Bachmann
YAAI	F^-	thi-1 his-68 trp-45 mtl-2 xyl-7 malA-1 galK-35 fabA-2 str-118 $\lambda^R \lambda^-$	B. Bachmann
568	F^-	pyrD lac y-14 galU-95 mal λ^R str thi	J. Beckwith
G19	F^-	glut ade his thi poaA	H. Condamine

et al., 1950). Selection of recombinants was carried out on minimal medium supplemented with amino-acid or bases when necessary at concentration of 40 μg/ml, thiamine (1 μg/ml), potassium glutamate (2 mM). When they were provided as carbon source, proline was added at 0.6%, maltose and xylose at 10%.

Enzyme Assays

Alkaline phosphatase and aminopeptidase activities were determined in intact or toluenized cells suspensions as previously described (Lazdunski et al., 1975). Cell-free extracts were prepared from washed cells, broken in a French Pressure cell. The resulting suspension was centrifuged for 20 minutes at 40,000 *g* in a Sorvall RC2B centrifuge.

Mutagenesis

Strain 304 was used to look for mutants. Mutagenesis with ethyl methane sulfonate (EMS) was carried out as described by Condamine (1971). Mutagenesis with N-methyl-N′-nitro-N-nitrosoguanidine (NTG) was performed as previously described (Adelberg et al., 1965).

Conjugation

Mating between Hfr and F^- strains were carried out as Jacob and Wollman (1961). All of the Hfr strains were counterselected by streptomycin.

Transduction

Transduction with P_1 were performed according to Lennox (1955). To obtain *fabA*$^+$ transductants, the method described by Semple (1975) was employed.

Results

Isolation of Mutants which Lack Aminopeptidase

We have previously reported (Lazdunski et al., 1975a) that only one enzyme in *Escherichia coli* free extracts is capable of hydrolyzing L-alanine-p-nitroanilide or L-alanine-β-naphthylamide. This enzyme appears to be located near the cell surface and thus we could expect to obtain, by using a naphthol azo-dye technique, a fast and highly resolutive colony staining. This expectation was fully confirmed. L-alanine-β-naphthylamide and Fast Garnet GBC were employed as recently described (Miller et al., 1974). The colonies acquired a dark red color within 1 to 2 minutes. After mutagenization, non-staining mutants could be easily detected.

Many such independent mutants were obtained after mutagenesis of *E. coli* K12 (strain 304) either by N.T.G. or by E.M.S. Very little or no hydrolytic activity toward L-ala-p-nitroanilide was detected either in crude extracts or at a R_F of 0.61 (Lazdunski et al., 1975a) by specific gel staining after electrophoresis on 7% polyacrylamide gel in the various mutants. The percentage of activity compared to the wild type in 10 independent mutants is shown in Table 2. The highest percentage encountered is 15% in two strains, another strain had 11%, all others had no aminopeptidase activity. One of those low activity mutants had an increased generation time in minimal medium and not in rich medium. Similar mutants unable to hydrolyse the same substrate have been recently described in *Salmonella typhimurium* (Miller et al., 1974). Moreover, we have found that the aminopeptidase in this organism shows the same electrophoretic migration on polyacrylamide gel as in *E. coli*, and cross-reacts with antibodies raised against purified *E. coli* aminopeptidase (Murgier et al., 1976). Since Miller and Mackinnon (1974) have called the corresponding genetic locus *pepN*, we have chosen the same name.

Characterization of the Material Synthetized in the Mutants

Figure 1 shows double diffusion tests where extracts from various mutants were tested for precipitation against an anti-wild type aminopeptidase antiserum. All mutants screened contained cross-reacting material (Table 2). This indicates that none of them carries an extensive deletion or an early non-sens mutation for the *pepN* gene.

Table 2. Physiological properties of aminopeptidase mutants

Strains	Percentage activity[d]	Generation time (min)		CRM	Percentage of cotransduction with *pyrD* marker[c]
		Rich medium	Minimal medium		
304 WT	100	45	70	+	
9200[a]	0	48	68	+	41
9201[a]	15	45	70	+	40.6
9202[a]	0	45	70	+	–
9203[b]	0	43	70	+	38
9204[b]	11	48	70	+	–
9205[b]	15	45	65	+	42.3
9206[b]	0	43	68	+	37.5
9207[b]	0	45	70	+	43.3
9208[b]	0	45	70	+	43
9210[b]	0	45	125	+	40

[a] Isolated after mutagenesis with E.M.S.
[b] Isolated after mutagenesis with N.T.G.
[c] Strain 568 was the receptor.
[d] Measured on French Press extracts and toluenized cells.

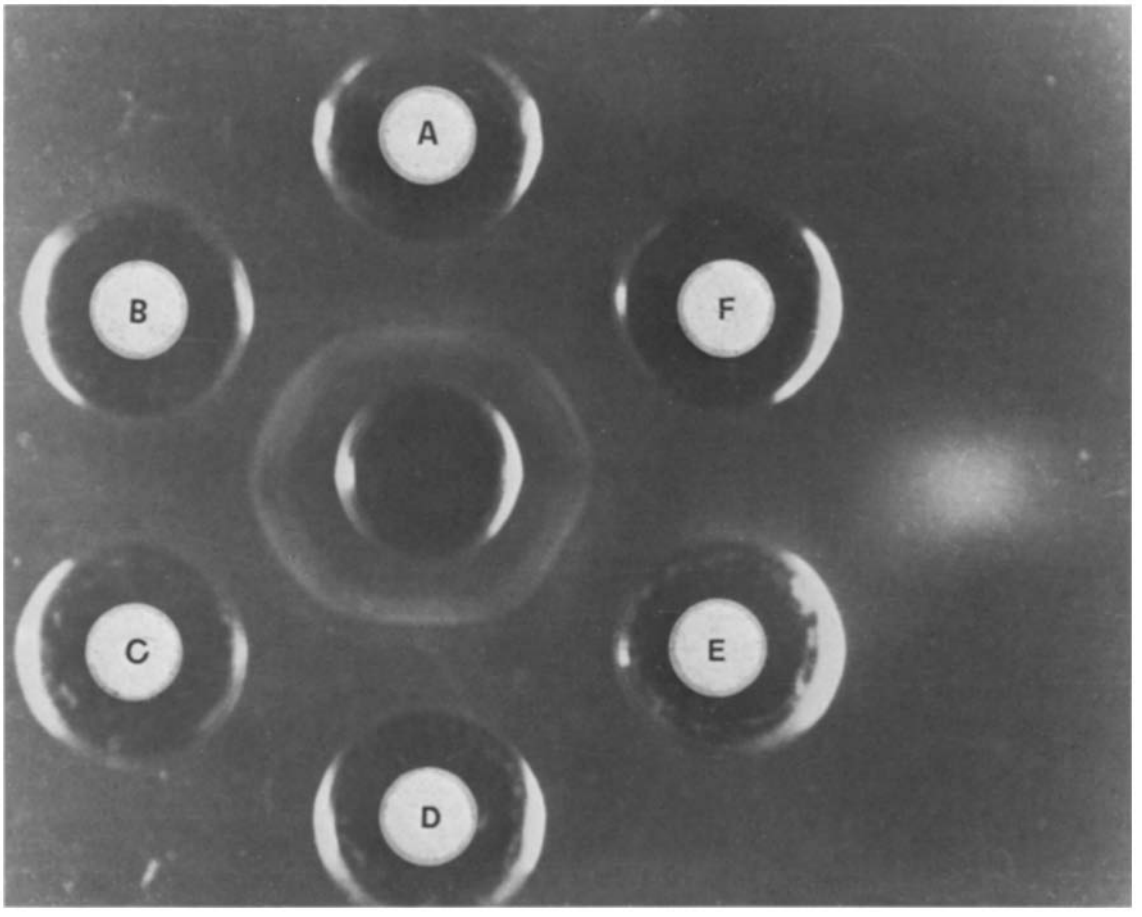
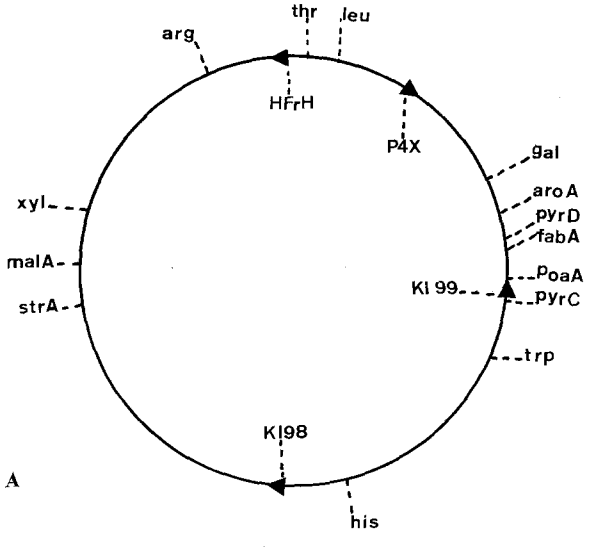


Fig. 1. Antibody-antigen precipitates in agar. Peripheral wells contained 150 µg of extracts of strains (A) 304, (B) 9204, (C) 9203, (D) 9202, (E) 9201, (F) 9200. The centre well contained 20 µl of antiserum



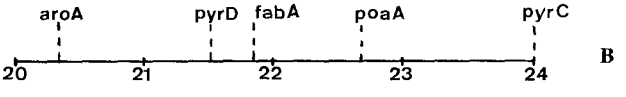


Fig. 2. A Genetic map of *E. coli* (Bachmann et al., 1976). Origins and directions of Hfr transfer are indicated by arrow heads. **B** Map positions of the markers used in transduction experiments

Mapping of pepN by Conjugation

Mating between three different Hfr and a F⁻ *pepN* mutant: strain 9200 (E.M.S. mutant of strain 304) were carried out. The origins and the directions of transfer of these Hfr are indicated in Figure 2A. In the first mating, the Hfr P4x was used as a donor and the F⁻ *pepN*⁻ strain 9200 unable to utilize mal-

Table 3. Mapping of *pepN* by conjugation

Relevant genotype of acceptor	Relevant genotype of donor	Selected markers and number analyzed		% unselected markers among selected recombinants				% of linkage	
				gal	pep	trp	his	$pep^+ trp^+$ / trp^+	$pep^+ his^+$ / his^+
9200	Hfr H	gal	201	100	65	6	1.5	100	
pep gal trp	$str^s pep^+$	trp	360	–	76.9	100	13.9		84
his str^R		his	180	–	53.3	61.1	100	82	
9200	KL983	his	108		7.4	24		30.7	
	xyl lac pep^+ str^s	trp	291		23.3		41.2		23.3
9200	KL99 $str^s pep^+$	trp	334		10.2		10.2		11.7

tose and xylose was used as a recipient. The thr^+, xyl^+ and mal^+ recombinants were then selected, counterselection for Hfr colonies was realized by growing the cells in the presence of streptomycin. Among these recombinants none was pep^+ which indicates that *pepN* locus is not located between *proB* and *strA*. The next mating was carried out with Hfr H as a donor and strain 9200 as a recipient. This latter is auxotrophic for tryptophan and histidine and it is unable to utilize galactose. Thus, after mating, the his^+, trp^+ and gal^+ recombinants were selected. The data presented in Table 3 show that the highest percentage of linkage was obtained between the pep^+ and trp^+ characters. Examination of the percentage of unselected markers among selected recombinants indicates a gradient of transmission in the order: *gal-pep-trp-his*. This suggestion is supported also by the fact that among the gal^+ recombinants selected, all of those that were trp^+ were also pep^+.

The transfer origin of Hfr KL983 is around 44 minutes in the chromosomal map and the direction of transfer is clockwise (Fig. 2). Using this Hfr and strain 9200 in crosses, we obtained respectively 23.3% and 7.4% of transmission of *trp* and *his* with pep^+. This suggests that *pepN* is closer to *trp* than to *his*. As well, 24% of the his^+ recombinants were trp^+ and 30.7% of these trp^+ were also pep^+. Among the 41.2% his^+ of the trp^+ recombinants, 23.3% of his^+ pep^+ strains were found. These results again suggest that pep^+ is located closer to *trp* than to *his*.

To check this point and precise the map position of *pepN*, the last conjugation was carried with Hfr KL99 that injects counterclockwise from about 22 minutes. The origin of Hfr KL99 was very close to *poaA*, between *poaA* and *pyrC* (Semple, 1975). Among the 334 trp^+ recombinants selected 10.2% and 10.2% respectively were found to be pep^+ and his^+. This result seems to be due to a preexisting population of episomes carrying distal region of the Hfr and thus pep^+ since we have found that a recombinant pep^+ (9200 × KL99) could transfer the poa^+ character in a poa^- strain.

It was thus suggested that the *pepN* mutation was around 20.5–22.5 minutes.

Determination of the Map Position of pepN by Transduction

P_1 mediated transductions of the pep^- character were investigated with two different recipients. With the strain YAA_1 which is $fabA^-$, among the $fabA^+$ recombinants 50% were found to be pep^-. With the strain 568 which is $pyrD^-$ as recipient, 40% of the recombinants were also pep^-. This transduction was carried out with most of the $pepN^-$ mutants that we have isolated. In all cases similar percentage were found, whether or not they showed a residual aminopeptidase activity. Thus, all these strains independently obtained carry mutations in the same gene. These data, as well as the fact that no co-transduction of pep^- with *pyrC* and *poaA* was obtained suggest that the *pepN* gene, in *Escherichia coli*, is located between 20.5 and 22.5 minutes on the chromosomal map. These results are schematized on Figure 2B.

Discussion

The electrophoretic migration on polyacrylamide gels (Murgier et al., 1976b) the specificity toward the various substrates tested and immunological cross-reaction (Murgier et al., 1976b) suggested that the aminopeptidase previously described in our laboratory (Lazdunski et al., 1975a) corresponds to the peptidase N recently found in *Salmonella typhimurium* (Miller et al., 1974). The map position that is described in this report strongly supports this hypothesis since

pepN locus was found to be near *pyrD* on the genetic map of *S. typhimurium* (Miller, 1975).

All of the mutants described probably carry a mutation in the aminopeptidase structural gene. However, regulatory mutants producing low levels of aminopeptidase might be encountered. Indeed, we have previously shown that the differential rate of synthesis of this enzyme is increased 4-fold when the bacterial growth is limited by inorganic phosphate (Lazdunski et al., 1975a). A simultaneous derepression of alkaline phosphatase, independent of the *phoR* and *phoB* regulatory gene, occurs under these growth conditions (Murgier et al., 1976a). Therefore, the production of alkaline phosphatase has been checked in all aminopeptidase mutants. We found a normal and well regulated level of alkaline phosphatase in these mutants. Thus, this reinforces the probability for all the mutations to be located in the structural gene of aminopeptidase. We are currently trying to isolate regulatory mutants to improve our understanding of the regulation by inorganic phosphate.

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