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pepM Is an Essential Gene in *Salmonella typhimurium*

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The *pepM* gene of *Salmonella typhimurium* codes for a methionine-specific aminopeptidase that removes N-terminal methionine residues from proteins. This gene was inactivated in vitro by the insertion of a DNA fragment coding for kanamycin resistance. The inactivated gene could not replace the wild-type chromosomal *pepM* gene unless another functional copy was present in the cell. The lethal effect of the *pepM* insertion was not a result of polarity on any gene downstream, nor was it affected by the presence or absence of other peptidases.

The synthesis of all bacterial proteins is thought to be initiated with *N*-formylmethionine. Before achieving their mature forms, however, nearly all proteins undergo removal of the formyl group and many proteins also lose the N-terminal Met residue. This process requires the sequential action of a formylase (1) and a Met-specific aminopeptidase. We have presented evidence that in *Salmonella typhimurium*, N-terminal Met removal is carried out by peptidase M, the product of the *pepM* gene (7). This gene has been cloned, and its complete nucleotide sequence has been determined (N. R. Movva et al., manuscript in preparation). It codes for an enzyme that is highly specific for N-terminal Met and for the amino acid that follows N-terminal Met in the peptide chain (7). This "second amino acid specificity" is consistent with the rules for Met removal that have been deduced from sequence comparison studies (8). A similar enzyme in *Escherichia coli* has been identified by Ben-Bassat and coworkers (3).

Although the removal of N-terminal Met seems to occur in all organisms, there are few proteins that are known to require such processing for function. We were interested, therefore, in determining whether N-terminal Met removal is necessary for survival.

(A preliminary report of these results was presented at the 88th Annual Meeting of the American Society for Microbiology, Miami Beach, Fla., 8 to 13 May 1988.)

The pBR328-derived plasmid pCM112 carries a 1.26-kilobase (kb) insert containing *pepM* with 0.19 kb of sequence upstream and 0.24 kb of sequence downstream. A DNA fragment encoding kanamycin resistance (Pharmacia GenBlock Kan^r casette) was inserted in vitro into the *Hpa*I site in the *pepM* coding region of pCM112 to generate pCM121. pCM121 carries a *pepM* gene interrupted by the Kan^r casette at a site approximately one-fifth of the distance between the N terminus and the end of the coding region. The mutation generated by this procedure was assigned the allele number *pepM200*::Kan. A strain carrying pCM121 lost the peptidase M overproduction phenotype normally conferred by pCM112 and the ability to use Met-Gly-Gly as a Met source (7) that results from this overproduction (Table 1).

The *pepM200*::Kan mutation was moved into the chromosome of a strain carrying a tandem duplication of the *pepM* locus to produce a heterozygous *pepM*$^+$/*pepM200*::Kan diploid. This was done by first transducing (with bacteriophage P22 HT 12/4 *int*3) the *pepM200*::Kan plasmid (pCM121) into a *polA* strain (10). This forces integration of the plasmid into the chromosome (4, 5) without loss of the chromosomal wild-type *pepM* allele. A transducing lysate prepared on this strain was used to transduce a *polA*$^+$ *pepM*$^+$/*pepM*$^+$ tandem chromosomal duplication strain (TN2563; 2), selecting only for Kan^r (i.e., for *pepM200*::Kan). Screening these transductants by replica plating identified a strain (1/686) that did not acquire the plasmid-encoded chloramphenicol resistance (Cam^r) marker. This strain (TN3027) was purified and characterized further. Southern hybridization experiments (Fig. 1) showed that TN3027 contained no pBR328 DNA and had indeed arisen by replacement of one of the chromosomal copies of *pepM* with the *pepM200*::Kan allele originally carried by the plasmid.

Several lines of evidence indicated that the *pepM200*::Kan mutation is lethal. (i) Kan^r haploid segregants were not produced by the *pepM*$^+$/*pepM200*::Kan heterozygous duplication strain. Tandem chromosomal duplications are genetically unstable because homologous recombination can occur between the two sides of the duplication (2). Heterozygous duplications produce haploid segregants that carry only one of the alleles originally present. If a mutation in one side of the duplication is lethal, segregants that lose the wild-type gene will not be observed. Of 297 potentially haploid segregants from TN3027, only two were Kan^r. These two represented possible haploid *pepM200*::Kan strains. On further testing, however, both of these strains were found to segregate Kan^s colonies. These strains were therefore not haploid *pepM200*::Kan but remained *pepM*$^+$/*pepM200*::Kan heterozygotes. No stable haploid Kan^r segregants were found. (ii) The *pepM200*::Kan mutation could not be transduced into haploid strains as either a selected or an unselected marker. When a transducing lysate on TN3027 (the *pepM*$^+$/*pepM200*::Kan duplication strain) was used to transduce a *pepM*$^+$ haploid and a *pepM*$^+$/*pepM*$^+$ duplication to Kan^r, only the duplication strain gave stable transductants (Table 2). Additional evidence for the haploid lethality of the *pepM200*::Kan mutation was obtained from crosses in which Kan^r was not directly selected. We carried out transduction crosses in which the donor was a strain (TN3030) carrying Tn*10*dCam (9) near (75% cotransducible with) *pepM200*::Kan (i.e., a *pepM*$^+$/*pepM200*::Kan *zae-1633*::Tn*10*dCam strain). Cam^r transductants were selected by using as recip-

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TABLE 1. Peptidase M levels[a]

Strain	Plasmid	Peptidase M sp act	Met-Gly-Gly utilization
TN1885	None	0.021	−
TN2950	pCM112 (*pepM*$^+$)	0.89	+
TN3004	pCM121 (*pepM200*::Kan)	0.023	−

[a] Peptidase M was assayed as described previously (7), using Met-Ala-Ser as a substrate. TN1885 and the two plasmid-containing strains derived from it are *leuBCD485 metA15 pepN90 pepA16 pepB11 supQ302* (*pepD proAB*) *pepP1 pepQ1 pepT1 pepE1*. Utilization of Met-Gly-Gly as a Met source was determined by spot tests on plates. Utilization of this peptide requires overproduction of peptidase M.

TABLE 2. P22 transduction crosses with selection for Kan^r

Donor	Recipient	No. of transductants
TN3027(*pepM200*::Kan/*pepM*$^+$)	TN2294 (*pepM*$^+$ haploid)	0[a]
TN3027	TN3026 (*pepM*$^+$/*pepM*$^+$ diploid)	357
TN3027	TN2950(pCM112)	400

[a] Two Kan^r colonies arose from this cross. Both were tested for stability and were found to produce Kan^s segregants when grown without selection for Kan^r. This indicates that these transductants contained heterozygous (*pepM*$^+$/*pepM200*::Kan) duplications and were not rare survivors that contained a single copy of the *pepM200*::Kan allele. The occurrence of such duplication transductants is expected as a result of the presence in the recipient population of spontaneous chromosomal duplications (2).

ient either a haploid *pepM*$^+$ strain or a *pepM*$^+$/*pepM*$^+$ duplication strain, and inheritance of Kan^r as an unselected marker was scored (Table 3). When the duplication strain was the recipient, Kan^r was inherited at the expected frequency. With the haploid recipient, however, none of the Cam^r transductants were Kan^r. As a control, a similar cross using as donor a strain (TN3029) carrying Tn*10*dCam near *pepM100* (i.e., a *pepM100 zae-1633*::Tn*10*dCam/*pepM200*::Kan strain) was carried out. (*pepM100* is an up promoter mutation with a scorable phenotype [7].) The two recipients inherited *pepM100* at the same frequency. All of these results provide very strong evidence that the *pepM200*::Kan allele is lethal.

It is conceivable that *pepM* is part of an operon and that the polarity of the Kan^r insertion on the expression of some downstream gene is responsible for the observed lethality. To test this possibility, we attempted to transduce *pepM200*::Kan into the chromosome of a strain carrying pCM112, a plasmid that carries the wild-type *pepM* allele and only about 0.24 kb of downstream sequence. The *pepM200*::Kan mutation could indeed be transduced into this strain at the expected frequency (Table 2). Strains carrying the *pepM200*::Kan mutation in the chromosome and pCM112 no longer lost the plasmid when grown without selection for plasmid-coded antibiotic resistance (data not shown). The lethal effect of the *pepM200*::Kan mutation must, therefore, be due to inactivation of *pepM* and not to loss of some downstream gene.

All of the experiments described here have used strains lacking a number of broad-specificity aminopeptidases, several of which can hydrolyze peptides with N-terminal Met (6). We considered the possibility that loss of *pepM* might be lethal only in such a peptidaseless strain. Perhaps one of the broad-specificity enzymes might be able to substitute for peptidase M in protein maturation. We attempted to introduce the *pepM200*::Kan mutation into a *pepM*$^+$ haploid strain (TN1379) that was wild type in all *pep* loci. The

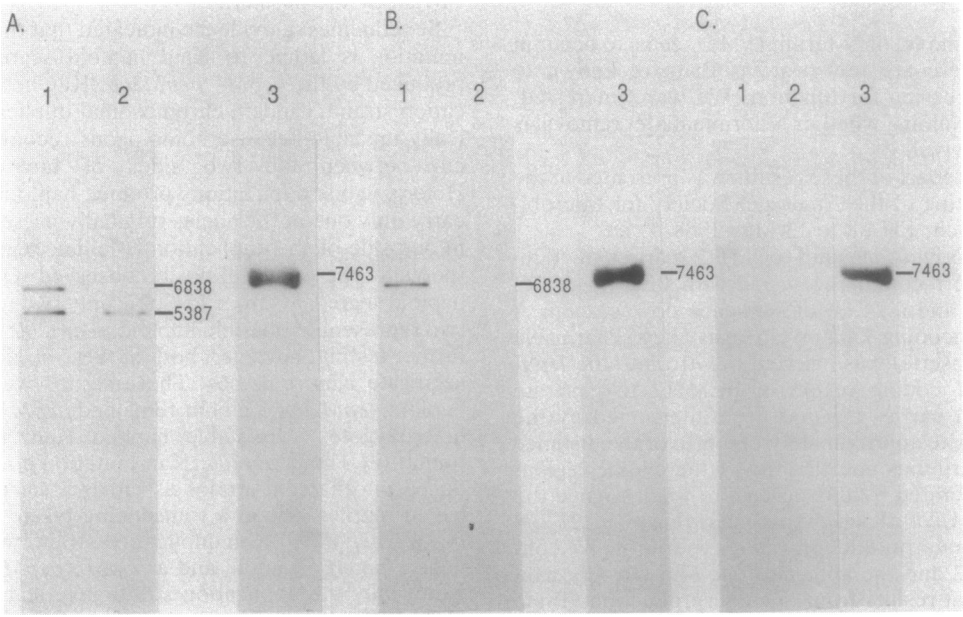

FIG. 1. Southern hybridization analysis. DNAs from TN3027 (*pepM*$^+$/*pepM200*::Kan) (lanes 1), TN1885 (*pepM*$^+$) (lanes 2), and purified pCM112 plasmid (lanes 3) were digested with *Bgl*II (A and B) or *Eco*RV (C) and probed with the following labeled restriction fragments: (A) a 934-base-pair *Nco*I-*Bam*HI fragment (containing only *pepM* sequence) from pRM4; (B) a 1,454-base-pair *Hin*cII fragment (containing only *aphI* [Kan^r casette] sequence) from pUC4-K; and (C) a 4,749-base-pair *Hin*dIII-*Eco*RV fragment (containing only pBR328 DNA) from pBR328. None of these restriction enzymes cuts in *pepM* or *aphI*. The calculated difference in size between the two *pepM*-hybridizing fragments from the duplication strain is 1,451 bases. The Kan^r casette insert is 1,454 bases long. Standards for determining fragment sizes (base pairs) were from a *Bst*EII digest of lambda DNA.

TABLE 3. P22 transduction crosses with selection for Cam^r

Donor	Recipient	Unselected marker	No. tested	No. (%) with unselected marker
TN3029	TN1885 (*pepM*$^+$ haploid)	*pepM100*	79	41 (52)
TN3030	TN1885	*pepM200*::Kan	176	0 (0)
TN3029	TN3026 (*pepM*$^+$/*pepM*$^+$ diploid)	*pepM100*	34	14 (41)
TN3030	TN3026	*pepM200*::Kan	47	32 (68)

pepM200::Kan gene could not be introduced into the *pep*$^+$ strain, showing that the *pepM200*::Kan mutation is lethal in both *pep*$^+$ and *pep* mutant strains and that none of the other peptidases can substitute for peptidase M.

N-terminal Met removal appears to be a universal property of living things, and the rules that determine which proteins undergo the process seem to be the same for all organisms studied (8). It is perhaps not surprising, therefore, to find that the ability to carry out the process is required for cell viability. It will be interesting to learn which proteins require N-terminal Met removal for function. When conditional mutations in *pepM* are isolated, it should be possible to approach this question.

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