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Genetic Characterization and Molecular Cloning of the Tripeptide Permease (*tpp*) Genes of *Salmonella typhimurium*

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Of the three bacterial peptide transport systems only one, the oligopeptide permease, has been characterized in any detail. We have now isolated *Salmonella typhimurium* mutants deficient in a second transport system, the tripeptide permease (Tpp), using the toxic peptide alafosfalin. Alafosfalin resistance mutations map at three loci, the gene encoding peptidase A (*pepA*) and two transport-defective loci, *tppA* and *tppB*. Locus *tppA* has been mapped to 74 min on the *S. typhimurium* chromosome, cotransducible with *aroB*, and is a positive regulator of *tppB*. Locus *tppB* maps at 27 min in the cotransduction gap between *purB* and *pyrF*. We cloned *tppB*, the structural locus for the tripeptide permease. Two simple methods are described for mapping the location of cloned DNA fragments on the chromosome of *S. typhimurium*.

Peptides serve an important nutritional role in the growth of many bacterial species (29, 30). In *Salmonella typhimurium* and *Escherichia coli* three distinct systems mediate the transport of peptides across the cytoplasmic membrane. These systems have overlapping specificities and between them will handle any peptide containing up to five amino acid residues, more or less independently of the nature of their amino acid side chains. Little or no affinity is shown towards free amino acids. The most completely characterized of the three peptide transport systems is the oligopeptide permease (Opp). Opp is encoded by four genes, *oppA*, *oppB*, *oppC*, and *oppD* (15, 16), which map as a single operon near *trp*, at 34 min on the *S. typhimurium* chromosome and 27 min in *E. coli* within the region inverted between the two species (35). Opp requires the function of an abundant periplasmic protein encoded by *oppA* (14) and will transport essentially any peptide containing up to five amino acids (32). The second peptide transport system, the dipeptide permease (Dpp), is relatively specific for dipeptides (28). Mutations in *dpp* have been isolated by a variety of selection procedures and map at 80 min on the *S. typhimurium* chromosome (unpublished data).

In addition to Opp and Dpp, a third peptide transport system was identified some years ago in *E. coli* (6, 27). This system was initially called the specialized or restricted system since its substrate specificity was believed to be limited to certain hydrophobic tripeptides. However, the use of more sensitive assays has shown that this system will transport a wide range of di- and tripeptides (J. W. Payne, personal communication), albeit with a preference for those containing hydrophobic amino acids. It therefore seems inappropriate to refer to this system as the restricted peptide permease. We propose the description tripeptide permease (Tpp), encoded by the *tpp* gene(s). This distinguishes the system from Dpp: whereas Dpp and Tpp each transport both di- and tripeptides, dipeptides are handled preferentially by Dpp and tripeptides by Tpp. Tpp previously was believed to serve a relatively minor role in peptide uptake when compared with Opp. However, in the accompanying paper (17) we show that under anaerobic conditions Tpp is specifically induced, becoming a major route for peptide uptake.

Mutants defective in Tpp have not previously been reported. In this paper we describe the isolation and characterization of *tpp* mutations in *S. typhimurium*, selected by using the peptide antibiotic alafosfalin (1). Three separate genetic loci were found to confer alafosfalin resistance; the gene for peptidase A (*pepA*) and two transport-defective loci which we named *tppA* and *tppB*. We mapped *tppA* and *tppB* to 74 and 27 min, respectively, on the *S. typhimurium* chromosomal map. We also report the molecular cloning of *tppB*, which encodes the anaerobically induced structural components of the tripeptide permease.

MATERIALS AND METHODS

Bacteria, phage and media. All strains used in this study are derivatives of *S. typhimurium* LT2. Their genotypes are listed in Table 1. Cells were grown at 37°C with aeration in LB medium (26) or on LB agar plates unless otherwise stated. Minimal glucose (MG) liquid medium and agar plates were based on the E medium of Vogel and Bonner (described in reference 34). Tetracycline (Tet), kanamycin (Kan), streptomycin (Str), and chloramphenicol (Cml) were used at 10, 50, 40, and 12.5 μg ml^{-1}, respectively. Amino acid supplements, when required, were used at 0.4 mM.

Transductions were carried out by using a high-transducing derivative of phage P22 *int-4* (36), as described by Roth (34).

Isolation of Tn*5* and Tn*10* insertions. Random Tn*5* insertions into the chromosome of CH231 were isolated by transduction of this strain to Kan^r, using a P22 lysate grown on strain TT3416 as described previously (7). Strain TT3416 carries Tn*5* on an F factor. Upon introduction into a recipient cell, stable Kan^r transductants can only arise by transposition of Tn*5* from the F factor onto the chromosome. A population of at least 10^4 independent Kan^r transductants was pooled and washed twice with E medium before further selections were applied. Random Tn*10* insertions in strain CH384 were obtained by using the defective P22::Tn*10* system described by Kleckner et al. (21). Again, at least 10^4 independent insertions were pooled and washed twice with E medium before further selection.

Conjugations. Hfr strains with the point of origin of transfer in *tppA* or *tppB* were constructed according to the method of Chumley et al. (8), using homology between Tn*5* or Tn*10* insertions to direct integration of F'ts114 *lac* into the chromosome. Selection for F'ts114 *lac* transfer was carried out on carbon- and nitrogen-free plates (12) containing 0.5%

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TABLE 1. Bacterial strains

Organism and strain	Genotype	Construction or source
S. typhimurium		
CH231	Δ*oppBC250 leu-1151*::Tn*10*	This laboratory
CH330	*str-1216*	Str^r LT2
CH331	*zcg-1703*::Cml^r (Cml^r is 90% linked to *tppB*)	Integration of pCH4 into the LT2 chromosome
CH332	*zcg-1704*::Cml^r (Cml^r is 90% linked to *tppB*)	Integration of pCH4 into the LT2 chromosome
CH333	*zcg-1705*::Cml^r (Cml^r is 90% linked to *tppB*)	Integration of pCH4 into the LT2 chromosome
CH338	*ompD159*::Tn*10*	P. H. Makela
CH345	Δ*oppBC250 leu-1151*::Tn*10 tppB5*::Tn*5*	This study
CH346	Δ*oppBC250 leu-1151*::Tn*10 tppB6*::Tn*5*	This study
CH347	Δ*oppBC250 leu-1151*::Tn*10 tppB7*::Tn*5*	This study
CH348	Δ*oppBC250 leu-1151*::Tn*10 tppB8*::Tn*5*	This study
CH349	Δ*oppBC250 leu-1151*::Tn*10 tppB9*::Tn*5*	This study
CH356	Δ*oppBC250 tppB16*::Tn*10*	This study
CH384	Δ*oppBC250* Δ*leu-3051*	Tet^s derivative of CH231
CH473	Δ*oppBC250 leu-1151*::Tn*10 tppB62*::Tn*5*	This study
CH475	Δ*oppBC250 leu-1151*::Tn*10 tppB64*::Tn*5*	This study
CH511	Δ*oppBC250 leu-1151*::Tn*10 tppA66*::Tn*5*	This study
CH512	Δ*oppBC250 leu-1151*::Tn*10 tppA67*::Tn*5*	This study
CH563	*purB85 ara-9 hisF8539*::Tn*10*	Recipient, SA2004; donor P22 lysate, TT29
CH564	*pyrF146 leu-500 hisF8539*::Tn*10*	Recipient, DW50; donor P22 lysate, TT29
CH565	*pyrC138 hisF8539*::Tn*10*	Recipient, *pyrC138*; donor P22 lysate, TT29
CH659	Δ*pyrC73 tppA66*::Tn*5*	Recipient, Δ*pyrC73*; donor P22 lysate, CH511
CH660	*purB85 ara-9 tppA66*::Tn*5*	Recipient, SA2004; donor P22 lysate, CH511
CH661	*pyrF146 leu-500 ompD159*::Tn*10 tppA66*::Tn*5*	Recipient, CH375
CH662	Δ*oppBC250* Δ*leu-3051 tppA66*::Tn*5 argG1828*::Tn*10*	This study
CH680	Δ*oppBC250* Δ*leu-3051 pepA201*::Tn*10*	This study
CH681	Δ*oppBC250* Δ*leu-3051 pepA202*::Tn*10*	This study
CH682	Δ*oppBC250* Δ*leu-3051 pepA203*::Tn*10*	This study
CH683	Δ*oppBC250* Δ*leu-3051 pepA204*::Tn*10*	This study
CH684	Δ*oppBC250* Δ*leu-3051 pepA205*::Tn*10*	This study
CH685	Δ*oppBC250* Δ*leu-3051 tppB16*::Tn*10*	This study
CH687	Δ*oppBC250* Δ*leu-3051 tppB53*::Tn*10*	This study
CH692	Δ*oppBC250* Δ*leu-3051 tppB58*::Tn*10*	This study
CH695	*tppB16*::Tn*10*	LT2 transduced to Tet^r with CH685 as donor
S294	*argI539*	K. E. Sanderson
SA486	*serA13 rfa-3058* HfrK3	K. E. Sanderson
SA534	*serA13 rfa-3058* HfrK4	K. E. Sanderson
SA722	*serA15 pur-268* HfrK10	K. E. Sanderson
SA2004	*purB85 ara-9*	K. E. Sanderson
SA2016	*aroB74 ara-9*	K. E. Sanderson
SGSC218	*purB*::Tn*10*	K. E. Sanderson
TA1885	*polA2 zig-214*::Tn*10*	C. Higgins and G. F.-L. Ames, unpublished data
TN996	Δ*(leuBCD)485 pepA1 pepN10 pepB1 pepD1 zjh-829*::Tn*10*	C. G. Miller
TN1009	*dcp-1 zxx-845*::Tn*10*	C. G. Miller
TN1301	Δ*(leuBCD)485 pepA16 pepB11* Δ*supQ302(proAB-pepD) pepP1 pepQ1*	C. G. Miller
TN1302	Δ*(leuBCD)485 pepB11* Δ*supQ302(proAB-pepD) pepN90 pepP1 pepQ1*	C. G. Miller
TN1303	Δ*(leuBCD)485 pepA16* Δ*supQ302(proAB-pepD) pepN90 pepP1 pepQ1*	C. G. Miller
TN1304	Δ*(leuBCD)485 pepA16 pepB11 pepN90 pepP1 pepQ1*	C. G. Miller
TN1420	Δ*(leuBCD)485 pepN90 pepA16 pepB11 pepP1 pepQ1 supQ302(proAB-pepD) pepT1 metE338 zie-882*::Tn*5*	C. G. Miller
TN1910	*pepT7*::Mud1 *oxrA1 zda-888*::Tn*10* (15% linked to *oxr*)	C. G. Miller
TT142	*argG1828*::Tn*10*	J. Roth
TT460	*pyrB692*::Tn*10*	J. Roth
TT627	*strA1 pyrC7*/F'ts114 *lac*$^+$ *zzf-20*::Tn*10*	J. Roth
TT628	*strA1 pyrC7*/F'ts114 *lac*$^+$ *zzf-21*::Tn*10*	J. Roth
TT629	*strA1 pyrC7*/F'ts114 *lac*$^+$ *zzf-22*::Tn*10*	J. Roth
TT3401	*pyrB655*/F'114 *lac*$^+$ *zzf-696*::Tn*10*	J. Roth
TT3406	*pyrB655*/F'114 *lac*$^+$ *zzf-701*::Tn*5*	J. Roth
TT3408	*pyrB655*/F'114 *lac*$^+$ *zzf-703*::Tn*5*	J. Roth

Continued on following page

TABLE 1.—*Continued*

Organism and strain	Genotype	Construction or source
E. coli K-12		
HB101	*hsdS20*(r_B^- m_B^-)*recA13 ara-14 proA2 lacY1 galK2 rpsL20* (Sm^r) *xyl-5 mtl-1 supE44*	(15)
CH212	*hsdS20*($r_B^-m_B^-$)*recA13 ara-14 proA2 lacY1 galK2 rpsL20* (Sm^r) *xyl-5 mtl-1 supE44 oppA462*	(15)
CH213	*hsdS20*(r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20* (Sm^r) *xyl-5 mtl-1 supE44 oppA463*	(15)
KL708	*leuB6 hisG1 recA1 argG6 metB1 lacY1 gal-6 malA1*(λ^r) *xyl-7 mtl-2 rpsL104 tonA2 tsx-1 supE44*/F'141	B. Bachmann
MAF1/JC1553	*leuB6 hisG1 recA1 argG6 metB1 lacY1 gal-6 malA1*(λ^r) *xyl-7 mtl-2 rpsL104 tonA2 tsx-1 supE44*/F'140	B. Bachmann

lactose as the sole carbon source and 10 mM ammonium chloride as the nitrogen source.

Donor and recipient strains for conjugational matings were grown in LB medium to an optical density at 650 nm of 0.4. The Hfr donor and F^- recipient cells were mixed in a ratio of 1:10 and after 60 min of incubation at 37°C were concentrated by centrifugation and spread on the appropriate selective plates. In cases where antibiotic selection against the donor was unnecessary, matings were carried out directly on appropriately supplemented MG plates (25). Interrupted matings were carried out by using a streptomycin-resistant derivative of LT2 (strain CH330) as the recipient. During liquid matings in LB medium, 0.5-ml samples were withdrawn at appropriate time intervals, and mating was interrupted by diluting the samples into 5 ml of ice-cold streptomycin (80 μg ml^{-1}) and vortexing for 5 min. Cells were left standing on ice for a further 20 min before being concentrated and spread on selective plates. F′ episome transfers were carried out similarly, except that the donor cells were grown in selective medium to ensure retention of the F factor. Matings for F′ transfer were for 6 h at 37°C in liquid medium with a 1:1 ratio of donor to recipient cells.

Identification of genotypes and phenotypes. Amino acid auxotrophy was tested by radial streaking on MG plates around filter paper disks impregnated with the appropriate amino acid (1 μmol). Mutations in *opp* were identified by resistance to triornithine, as previously described (15). The ability of auxotrophs to utilize peptides as the sole source of a required amino acid was determined by radial streaking around a disk containing 10 μmol (3.3 μmol for trileucine) of the appropriate peptide. Alafosfalin and bacilysin resistance or sensitivity was tested by radial streaking on MG plates around a disk containing 0.25 mg of alafosfalin or 0.5 mg of bacilysin. To obtain a semiquantitative measure of peptide utilization or antibiotic inhibition, 2×10^8 washed cells were plated on a minimal agar plate, and a filter disk of the compound to be tested was placed in the center. Zones of growth or inhibition were measured after 16 h of incubation at 37°C.

DNA isolation and characterization. Restriction endonucleases and T4 DNA ligase were purchased from Bethesda Research Laboratories or New England Biolabs, Inc., and used in conditions recommended by the manufacturers. *S. typhimurium* chromosomal DNA was isolated from exponentially growing cells as described previously (3). Plasmid DNA was purified from cleared lysates by cesium chloride density gradient centrifugation (9). A bank of chromosomal DNA in the plasmid vector pBR322 was constructed by digestion of chromosomal DNA and vector DNA with *Bam*HI. The digested DNA samples were extracted with an equal volume of chloroform-isoamyl alcohol (24:1) and precipitated with ethanol. Subsequently, the vector DNA was treated with calf-intestinal alkaline phosphatase (Boehringer) as described by Manatis et al. (23). The vector and chromosomal DNA were mixed and ligated for 12 h at 16°C in a total volume of 40 μl containing 1 mM ATP, 66 mM Tris (pH 7.6), 6.6 mM $MgCl_2$, and 10 mM dithiothreitol. The ligated DNA was transformed into *E. coli* 294, selecting for Amp^r colonies. More than 95% of the Amp^r derivatives were Tet^s, showing that the great majority of plasmids contained an inserted DNA fragment. Six thousand independent Amp^r colonies were pooled, diluted to 10^8 cells ml^{-1}, and grown to an optical density at 660 nm of 1.0, and plasmid DNA was isolated after 16 h of amplification with chloramphenicol (150 μg ml^{-1}). Transformations of $CaCl_2$-treated cells were carried out as described by Lederberg and Cohen (22), except that the $MgCl_2$ wash was omitted.

RESULTS

Isolation of *tpp* mutations. The peptide antibiotic alafosfalin must enter the cell to exert its toxic effect (1). In strains deleted for the *opp* genes, alafosfalin was found to remain toxic, implying that its uptake is mediated by one or both of the two remaining systems, Dpp and Tpp. Thus, one might be able to isolate mutations in *dpp* or *tpp* by selection for alafosfalin resistance. Preliminary experiments showed that spontaneous alafosfalin resistance mutations arise at a frequency of ca. 10^{-5}. Thus, it seemed probable that only a single mutational event is required for resistance. Alafosfalin-resistant mutants were selected on MG plates containing 80 μg of alafosfalin ml^{-1}. Tn*5* and Tn*10* insertions conferring alafosfalin resistance were isolated from populations of random transposon insertions in the Leu$^-$ strains CH231 and CH384, respectively. In all cases each derivative strain was shown by transduction to contain only a single transposon, and this insertion was shown to be responsible for the alafosfalin resistance phenotype. Eight independent alafosfalin resistance Tn*10* insertions and nine Tn*5* insertions were isolated and characterized (Table 1). When these insertions were mapped with respect to each other by cotransduction, they were found to fall into three distinct linkage groups. These three classes could also be distinguished phenotypically (Table 2). Class I was fully resistant to alafosfalin but was not defective in peptide uptake: utilization of a variety of peptides as the sole source of a required amino acid was unimpaired. These mutations were subsequently shown to be lesions in *pepA* (see below). Classes II and III were both defective in their ability to utilize a range of tripeptide substrates. Although Table 2 shows only the results for two leucine-containing tripeptides, Leu-Leu-Leu and Leu-Gly-

TABLE 2. Phenotypes of alafosfalin resistance mutations[a]

Strains	Growth inhibition zone (cm) with:		Growth zone (cm) with:		
	Alafosfalin	Bacilysin	Leucine	Leu-Leu-Leu	Leu Gly-Gly
Parental strain (*pepA*$^+$ *tpp*$^+$)	2.0	2.5	2.7	1.5	2.5
Class I (*pepA*)	0	2.5	2.7	1.5	2.5
Class II (*tppA*)	0.4	2.5	2.7	0.6	1.0
Class III (*tppB*)	0	2.0	2.7	0	0

[a] Zones of growth or growth inhibition were determined as described in the text. The strains used were as follows: CH231 and CH384 (parental strains), CH680 through CH684 (class I), CH511 and CH512 (class II), and CH345 through CH349, CH685, CH687, and CH692 (class III).

Gly, the ability of appropriate peptides to satisfy several other auxotrophic requirements was also impaired (data not shown). The utilization of free amino acids was unaffected. Because the leucine-containing (and other) peptides can each be cleaved by a range of independent peptidases (24, 25), the defects are presumably at the transport level. These class II and class III loci are designated *tppA* and *tppB*, respectively. Mutations in *tppA* and *tppB* could be distinguished phenotypically in three ways. First, whereas *tppB* mutants were completely resistant to alafosfalin, *tppA* mutants were only partially resistant, even though the genetic lesions were due to transposon insertions. Second, *tppA* mutations, unlike mutations in *tppB*, only resulted in a partial defect in the utilization of trileucine. Third, spontaneous bacilysin resistance mutations arise in *tppB* strains at a frequency of ca. 10^{-5} but in tppA strains at a frequency of $<10^{-9}$. This is due to the fact that the toxic peptide bacilysin (18, 19) is transported into cells by both Tpp and Dpp (Table 2; unpublished data): only in strains totally defective for Tpp function can a single mutational event (in *dpp*) confer bacilysin resistance. These three phenotypic differences indicate that whereas transposon insertions in *tppB* are totally defective in Tpp function, *tppA* insertion mutations still allow residual transport through this system. This suggests that *tppA* may play a regulatory role, rather than encoding the structural genes for Tpp. In the accompanying paper (17) we show that *tppA* does indeed encode a positive regulator of *tppB*.

Mutations in *pepA* confer alafosfalin resistance. It seemed most likely that alafosfalin resistant mutants unaffected in peptide uptake (class I) were deficient in peptidase activity, since it is known that alafosfalin must be hydrolyzed before it can exert its toxic effects (1). Although *S. typhimurium* contains a range of peptidases of overlapping specificity, it is possible that, because of its unusual structure, alafosfalin is a substrate for just one of these enzymes. We therefore tested a series of strains constructed by C. G. Miller, each deficient in all but one of the major peptidases. All strains deficient in *pepA* (TN1420, TN1301, TN1303, and TN1304; see Table 1 for genotypes) were alafosfalin resistant, whereas the single strain proficient for *pepA* (TN1302) was found to be alafosfalin sensitive. Thus, *pepA* appears to be the only *S. typhimurium* peptidase which can cleave alafosfalin. To determine whether the class I alafosfalin resistance mutations were in *pepA*, the lesions were mapped. The class I mutations were each shown to be cotransducible with *pyrB*

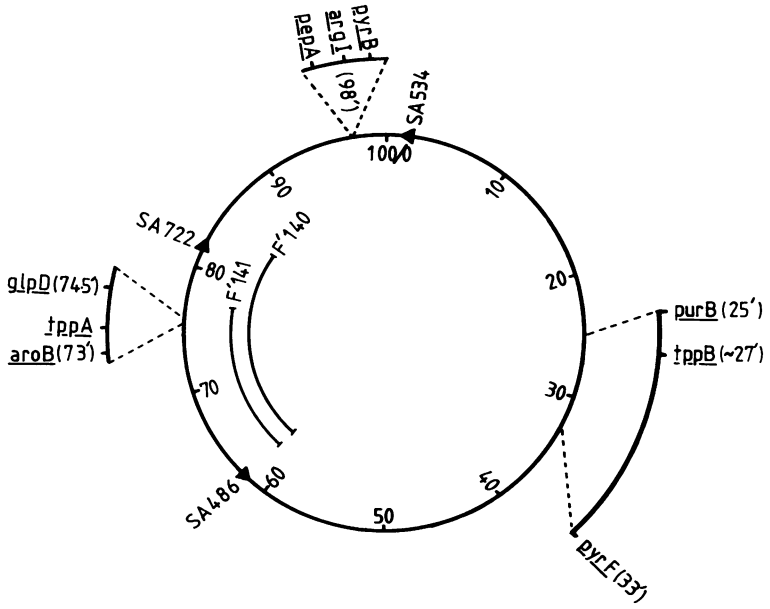

FIG. 1. Map of the *S. typhimurium* chromosome. The *S. typhimurium* chromosome is divided into 100 map units. The locations of *tppA*, *tppB*, and *pepA* with respect to adjacent genes are shown in the expanded sections. The points of origin and direction of transfer of Hfr strains used to map *tpp* are shown by the arrowheads on the chromosome. The portions of the chromosome covered by F'140 and F'141 are also shown.

(2%) and *argI* (38%) and therefore map in the same position as *pepA* (Fig. 1). As final confirmation of the identity of *pepA* and the class I mutations, TN1302 (*leu pepA' pepB pepD pepN* was transduced to Tet^r with P22 grown on strain TN996 (containing a Tn*10* transposon linked to a *pepA* point mutation). A proportion (35%) of the Tet^r derivatives were unable to utilize trileucine, showing that the *pepA* mutation has been introduced into these transductants (as *pepA* is the only functional peptidase in strain TN1302 able to cleave trileucine). All trileucine nongrowers simultaneously became alafosfalin resistant, confirming that *pepA* is responsible for alafosfalin hydrolysis.

Map location of *tppB*. A series of Hfr strains (SA486, SA534, and SA722; Fig. 1) carrying a Tn*10* insertion in *tppB* were constructed by transduction to Tet^r with CH356 as the donor. Each of these Hfr strains was mated with several different auxotrophic recipients, and the prototrophic recombinants were scored for inheritance of the Tn*10* transposon. Matings with *pyrC* and *trp* strains gave the highest proportion of Tet^r recombinants, indicating that *tppB* is located between *pyrC* (23 min) and *trp* (34 min; data not shown. To confirm and extend these observations, Hfr strains were constructed with the point of origin of transfer in *tppB*, by using Tn*5* and Tn*10* insertions in *tppB* to promote F' integration. By using appropriate F'ts *lac* episomes, Hfr strains were constructed such that transfer of chromosomal material was in either of the two possible directions. Strains CH349 and Ch356 were used to direct F'ts *lac*::Tn*5* or F'ts *lac*::Tn*10* insertions, respectively, and the resulting Hfr derivatives were mated with *pyrC*, *purB*, or *pyrF* strains and scored for prototrophic recombinants (Table 3). Hfr strains constructed from TT627 and TT629, in which the Tn*10* transposon on the F factor is in the A orientation, all transferred *pyrF* at high frequency. Hfr strains constructed from TT628, in which the transposon is in the opposite (B) orientation, transferred *pyrC* and *purB* at high frequency. Similar results were obtained with Tn*5*-mediated Hfr formation (Table 3). Thus, *tppB* must be located in the cotransduction gap (35) between *purB* at 25 min and *pyrF* at 33 min. Cotransduction with phage P22 showed that *tppB* was not linked to *pyrF*, *purB*, or any of the following genes also known to be located within the cotransduction gap: *ompD* (P. H. Makela, personal communication), *pepT* (37), *dcp* (39), and *fnr/oxrA* (17). To obtain a more accurate location within this cotransduction gap, the Hfr strain SA534 was transduced to *tppB*::Tn*5* with a P22 lysate grown on CH349 and was used for timed interrupted matings with CH330 (Str^r) as the recipient. The results (Fig. 2) show that *tppB* is transferred after 38 min, corresponding to a map location for *tppB* at 27 min (Fig. 1).

TABLE 3. Hfr mapping of *tppB*[a]

Hfr	Transposon on F'	Orientation	No. of prototrophic recombinants with:		
			pyrC	*purB*	*pyrF*
CH356/TT627	Tn*10*	A	5	10	165
CH356/TT629	Tn*10*	A	3	5	146
CH356/TT628	Tn*10*	B	222	217	10
CH349/TT3408	Tn*5*	A	70	71	1,226
CH349/TT3401	Tn*5*	B	1,164	1,135	90
CH349/TT3406	Tn*5*	B	1,175	1,244	84

[a] Hfr strains with the point of origin of transfer in *tppB* were constructed by using CH356 (*tppB*::Tn*10*) or CH349 (*tppB*::Tn*5*) and the indicated F'ts *lac* strains according to the method of Chumley et al. (8). The orientation of the transposon on F' is indicated. Each figure is the average of results obtained with at least three independently isolated Hfr strains. The recipients used were CH659 and CH565 (*pyrC*), CH660 and CH563 (*purB*), and CH661 and CH564 (*pyrF*).

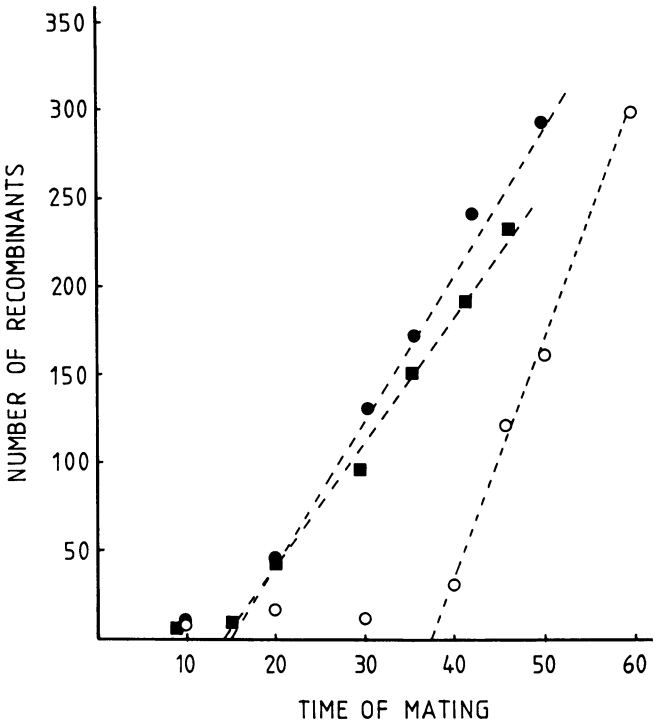

FIG. 2. Mapping by interrupted mating. Solid symbols indicate interrupted conjugations between SA486/*tppA*::Tn*5*(●) or SA722/*tppA*::Tn*5*(■) with CH330 as the recipient. Str^r Kan^r recombinants were scored. Open circles show mapping of *tppB* with SA534/*tppB*::Tn*5* as the donor and CH330 as the recipient, again scoring for Str^r Kan^r recombinants.

Mutants unable to utilize Met-Ala-Ser have been isolated in a multiply peptidase-deficient background (*pepN pepA pepB pepD pepP pepQ*) and shown to be defective in the uptake of Met-Ala-Ser (K. L. Strauch and C. G. Miller, personal communication). Strain TN1896 contains a Tn*5* transposon linked (60%) to one such mutation. We have found that this transposon is similarly linked to *tppB*, and in addition the lesion is complemented by plasmid pCH1 (see below). Thus, selection for Met-Ala-Ser nonutilization provides an alternate means of selecting mutations in *tppB*.

Mapping of *tppA*. The approximate map location of *tppA* was determined by transducing *tppA*::Tn*5* insertions into three Hfr strains (SA534, SA486, and SA722; Fig. 1) and mating these derivatives with strain CH330 (Str^r as the recipient. Kan^r Str^r recombinants were selected. Both strains SA486 and SA722 transferred the Kan^r marker at high frequency compared with SA534 (data not shown), indicating a location for *tppA* between the points of origin of transfer of SA486 and SA722 (63 and 83 min, respectively). *tppA* was more accurately located by interrupted matings with SA486 and SA722 as donors, each carrying a Tn*5* insertion in *tppA*. Figure 2 shows the results of these matings. Kan^r is transferred after 15 min by SA486 and after 14 min by SA722, indicating a map location at 73 to 74 min. To confirm this location Hfr strains with the point of origin of transfer in *tppA* were constructed. Used as donors, these

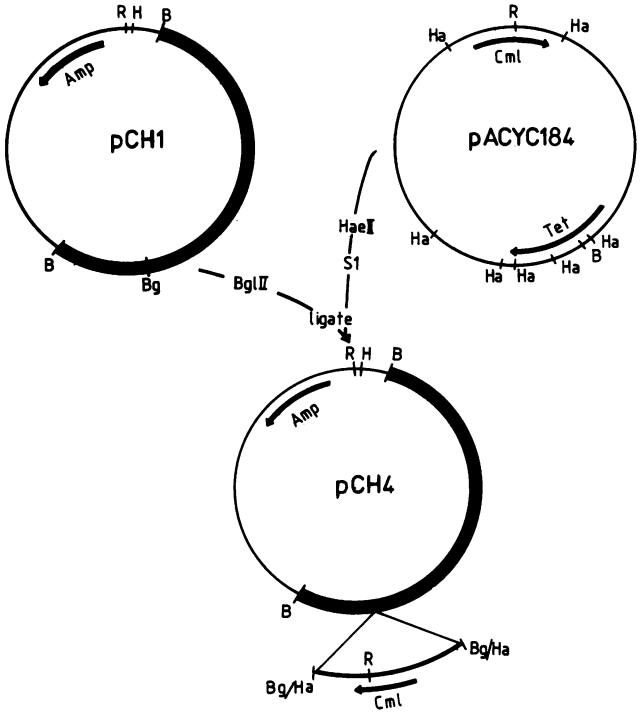

FIG. 3. Plasmid construction. Plasmid pCH1 contains a 6.4-kb chromosomal fragment (indicated by the solid black line) inserted into the *Bam*HI site of pBR322. This 6.4-kb fragment carries *tppB*. Plasmid pCH4 was constructed by inserting a 1.3-kb *Hae*II fragment from pACYC184, containing the chloramphenicol resistance gene, into the *Bgl*II site of pCH1. Plasmids pCH1 and pACYC184 were digested with *Bgl*II and *Hae*II, respectively, treated with S1 nuclease to remove the single-stranded extended ends, mixed, ligated, and transformed into strain 294. Plasmid DNA from Amp^r Cml^r Tet^s colonies was screened and characterized by restriction endonuclease digestion. Restriction endonuclease sites are abbreviated as follows: B, *Bam*HI; Bg, *Bgl*II; H, *Hin*dIII; Ha, *Hae*II; R, *Eco*RI.

strains showed that *tppA* is located between *aroB* at 73 min and *glp* at 74.5 min (data not shown). Subsequently the *tppA*::Tn*5* insertion in CH511 was shown to be 33% (33 of 100) cotransducible with *aroB*. Additional confirmation that *tppA* is located in this region of the chromosome is provided by the fact the *E. coli* F′ factors F′140 and F′141 (5) complement the *tppA* lesion. CH662 (*argG*::Tn*10* *tppA*::Tn*5*) was mated with *E. coli* strains carrying these F′ factors (KL708 and MAF1/JC1553), selecting for Arg$^+$ merodiploids. All such merodiploids became alafosfalin sensitive while still retaining the chromosomal *tppA*::Tn*5* insertion.

Alafosfalin is specifically transported by Tpp. We originally selected alafosfalin resistance mutations in Opp$^-$ strains since it seemed likely, based on the known specificity of Opp, that this peptide would enter cells via both Opp and Tpp. Subsequently, strain LT2 (Opp$^+$) was transduced to Tet^r with a P22 lysate of CH356 (*tppB*::Tn*10*) as the donor. All Tet^r transductants became alafosfalin resistant. Thus, alafosfalin cannot enter via Opp at a rate sufficient to confer toxicity.

Cloning of *tppB*. *E. coli* proline auxotrophs defective in Opp are unable to utilize Pro-Gly-Gly as a source of proline (14). A bank of 6,000 independent recombinant plasmids containing *Bam*HI fragments of the *S. typhimurium* chromosome inserted into pBR322 was constructed. Plasmid DNA was transformed into CH212 (Opp$^-$ Pro$^-$), with selection for Amp^r colonies able to utilize Pro-Gly-Gly (80 μM). Several such derivatives were isolated. Restriction analysis of plasmid DNA from each of these strains showed that each contained an identical 6.4-kilobase (kb) *Bam*HI insert (Fig. 3). Transformation of this DNA into CH212 gave a 1:1 correlation between Amp^r and Pro-Gly-Gly growth, confirming that the plasmid DNA, and not a spontaneous chromosomal mutation, was responsible for growth on Pro-Gly-Gly. Pro-Gly-Gly normally enters *E. coli* cells via Opp; Opp$^-$ strains are unable to utilize this substrate at a rate sufficient to confer growth of a proline auxotroph. We therefore anticipated that pCH1 would contain the cloned *opp* genes. However, nick-translated pCH1 DNA was found to hybridize identically in dot-blots with chromosomal DNA isolated from LT2 and from CH46, which carries a total deletion of the *opp* genes (data not shown). Thus, pCH1 must encode some other gene which in multicopy confers the ability to utilize Pro-Gly-Gly (Table 4). To ascertain whether or not pCH1 confers a general increase in peptide uptake, the plasmid was transformed into a variety of *E. coli* auxotrophs: in many cases growth on peptides containing the required amino acid was enhanced (data not shown). In addition, Table 4 shows that pCH1 renders cells sensitive to valine- or leucine-containing peptides, presumably causing an increase in peptide uptake, followed by release of free amino acids which, in sufficient concentrations, can interfere with isoleucine biosynthesis (38). To confirm this we showed that isoleucine can specifically overcome the toxic effects of leucine-containing peptides. In agreement with the hybridization data, the specificity of peptide uptake mediated by pCH1 was found to be unlike that of Opp. Thus, positively charged peptides were poorly utilized, whereas hydrophobic peptides were good substrates.

Integration of pCH1 into the chromosome. To identify the gene(s) encoded by pCH1 we had to map their location on the chromosome. This was achieved by forcing the plasmid to integrate into the chromosome by homologous recombination and mapping the point of integration. Two independent methods were used to direct plasmid integration.

(i) Integration by transduction. To provide a useful marker to follow integration, the chloramphenicol transacelytase

TABLE 4. Effect of pCH1 on growth and growth inhibition by peptides[a]

Strain (genotype)	Growth zone (cm) with:		Growth inhibiton zone (cm) with:		
	Pro-Gly-Gly	Pro	Val-Val-Val	Leu-Leu-Leu	Leu-Gly-Gly
HB101 (*opp*$^+$)	2.0	2.8	1.4	0.4	0.5
CH212 (*opp*)	0	2.7	0.8	0.2	0.2
CH212(pCH1) (*opp*)	1.2	2.6	3.3	1.5	1.8
CH213 (*opp*)	0	2.8	0.8	0.2	0.2
CH213(pCH1) (*opp*)	1.2	2.4	3.0	1.4	1.8

[a] Zones of growth or growth inhibition were determined as described in the text.

gene from pACYC184 was cloned into the *Bgl*II site of pCH1 (Fig. 3). The resultant plasmid, pCH4, was transformed into *S. typhimurium* LT2, selecting for Amp^r. Homogenotization between the plasmid and the chromosome will result in integration of the Cml^r marker at the point of homology between the chromosomal DNA inserted in pCH4 and the chromosome itself (Fig. 4). To identify the site of integration into the chromosome, the Cml^r marker must be transferred into a plasmid-free background. This was achieved by transduction. P22 phage grown on a pCH4-containing strain was used to transduce LT2 to Cml^r and 600 Cml^r colonies were replica plated onto LB-ampicillin plates. As P22 will carry either the plasmid or the chromosomal Cml^r marker, but not both, Cml^r derivatives arising by transfer of the plasmid would also be Amp^r, whereas those arising by transfer of a chromosomally incorporated copy of the Cml^r gene would be expected to be Amp^s. Three Cml^r Amp^s colonies (CH331, CH332, and CH333) were isolated and shown to be plasmid free: the Cml^r marker in these derivatives must have arisen by recombination between pCH4 and the chromosome (Fig. 4).

(ii) Integration in *polA* cells. Plasmids such as pBR322 which contain the ColE1 origin of replication are unable to replicate in *polA* cells (11, 20). Plasmid pCH1 was transduced into strain TA1885 (a *polA* derivative of LT2), constructed by use of a Tn*10* transposon which is 60% linked to the *polA* gene (C. F. Higgins and G. F.-L. Ames, unpublished data) selecting for Amp^r. The only way an Amp^r colony can arise with a *polA* recipient is by integration of the plasmid into the chromosome by homologous recombination (11). Three independent Amp^r colonies were selected for further analysis.

Mapping of pCH1. Strains CH331, CH332, and CH333 contain the Cml^r marker from pCH4 integrated into the chromosome and separated from the free plasmid by transduction (see above). The Cml^r marker from each of these three strains was transduced into various Hfr strains; interrupted matings showed that all three mapped to an identical location between 25 and 27 min on the *S. typhimurium* chromosome (data not shown). The three Amp^r strains derived by integration of pCH1 into the *polA* chromosome were each shown to be linked (>80%) by P22 transduction to the Cml^r marker of strain CH331. Thus, plasmid integration into the chromosome by either of the two methods described above occurs at the same point and must therefore be directed by homology between the plasmid and the chromosome rather than a random process. As *tppB* also maps in the *purB-pyrF* cotransduction gap, pCH1 was transduced into strain CH356 (*tppB*::Tn*10*); all Amp^r derivatives became alafosfalin sensitive and trileucine utilizers, showing that pCH1 complements *tppB* mutations. In addition, the Cml^r marker of CH331 was shown to be 90% linked to *tppB*::Tn*5* by P22 transduction. Thus, the chromosomal DNA cloned in pCH1 encodes *tppB*. Although a single copy of *tppB* is unable to transport sufficient Pro-Gly-Gly to satisfy a proline requirement, provision of the gene in multicopy enables this substrate to be utilized.

DISCUSSION

Using the toxic peptide alafosfalin, we were able to isolate and characterize mutations deficient in Tpp. These mutations have been mapped by a variety of techniques to two separate loci, *tppA* and *tppB*. Mutations at these loci can be distinguished phenotypically. Locus *tppA* is cotransducible with *aroB* at 74 min, and in the accompanying paper (17) is shown to encode a positive regulator of *tppB*. Locus *tppB* presumably encodes the structural components of Tpp and maps at 27 min, in the cotransduction gap between *purB* and *pyrF*. Locus *tppB* is not cotransducible with any of the other known genes in this region of the chromosome. We cloned the *tppB* genes into a multicopy plasmid. The cloned genes are shown to be *tppB* by the fact that the plasmid pCH1 complements *tppB* mutations and also, importantly, by the fact that the chromosomal DNA fragment of pCH1 maps to an identical location on the chromosome as does *tppB*. To map the location of the cloned DNA fragment on the chromosome, two techniques were used to force plasmid integration into the chromosome by homologous recombination. One method involves the separation of a chromosomally integrated copy of the plasmid from the independently replicating form by P22 transduction. The other relies on the inability of ColE1-based plasmids to replicate in *polA* strains unless integrated into the chromosome. *polA* strains have been used many times previously to direct plasmid integration into the chromosome (see references 11 and 13). The availability of a Tn*10* insertion linked to the *polA* mutation means that the *polA* mutation can be transferred into any *S. typhimurium* strain by a one-step procedure. These methods

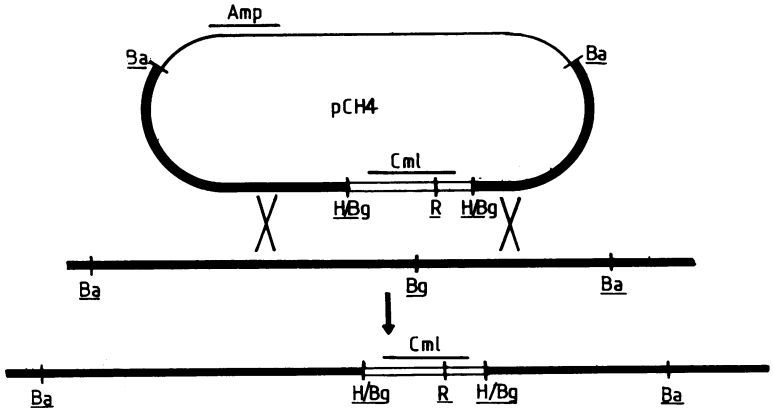

FIG. 4. Integration of pCH4 into the *S. typhimurium* chromosome. The 6.4-kb chromosomal insert of pCH4 is shown by the solid black line. The 1.3-kb fragment from pACYC184 (containing the Cml^r gene) which is inserted into the 6.4-kb fragment is shown by the white bar. The indicated double recombinational event between pCH4 and the homologous segment of the chromosome will result in transfer of the Cml^r marker of pCH4, but not the Amp^r gene, onto the chromosome. Restriction endonuclease sites are abbreviated as follows: Ba, *Bam*HI; Bg, *Bgl*II; R, *Eco*R1; H/Bg, hybrid *Hae*II-*Bgl*II site.

are generally applicable for mapping any cloned DNA fragment and should also be useful for transferring mutations between plasmid and chromosome.

Only one of the three bacterial peptide permeases, the Opp, has previously been characterized in any detail. Opp has generally been considered to show little specificity towards the carboxyl terminus of peptide substrates (31). However, the relatively poor uptake of alafosfalin via Opp shows that replacment of the carboxyl group by a phosphate moiety does have a profound effect on uptake by this transport system. The poor uptake of alafosfalin by Opp is in agreement with transport kinetic data (4). Because of its dipeptide structure, alafosfalin has often been assumed to enter cells via Dpp. However, we have shown here that the major route of uptake is in fact Tpp. This explains the unusual kinetics of alafosfalin uptake and inhibition observed by Atherton et al. (4) and is also in agreement with suggestions that an intact carboxyl group is necessary for uptake via Dpp (31). Because of the overlapping specificities of the three peptide transport systems, it is difficult to isolate mutations in *dpp* unless uptake via both Opp and Tpp is eliminated. The genetic characterization of Tpp therefore facilitates the isolation of mutations in *dpp* and the construction of strains proficient in any desired combination of the three transport systems (unpublished data). This will now permit a critical assessment of their substrate specificities and physiological roles in peptide uptake and excretion.

The broad specificity of the peptide permeases provides a mechanism by which normally impermeant molecules can gain entry into a cell conjugated in peptide form (2, 10). This concept, "illicit" transport, provides a rationale for the design of many peptide antibiotics (33). The synthetic peptide antibiotic alafosfalin (L-alanyl-L-aminoethyl phosphonic acid) must enter the cell and be cleaved to release L-aminoethyl phosphonic acid to exert its toxic effects, the inhibition of alanine racemase and consequently of cell wall biosynthesis (1). Although alafosfalin exhibits many ideal properties as an antibiotic, a major drawback is that resistant strains arise at high frequency. The results presented in this paper demonstrate why. First, despite the battery of intracellular peptidases, only one, peptidase A, is able to cleave alafosfalin. Secondly, although *S. typhimurium* and *E. coli* possess three peptide transport systems with overlapping specificities, significant uptake of alafosfalin is restricted to just one of these systems. Thus, a single mutation at any one of several independent loci can give rise to alafosfalin-resistant cells. A more complete consideration of the specificities of the bacterial peptidases and peptide transport systems is necessary for the design of more effective peptide antibiotics.

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ADDENDUM IN PROOF

Since submitting this paper and the accompanying paper, we found that *tppA* is indentical to the *ompB* locus, causing an $OmpF^-$ $OmpC^-$ phenotype. Thus, in addition to regulating the synthesis of porins, *ompB* is a positive regulator of *tppB* expression.

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