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Identification and Mapping of a Second Proline Permease in *Salmonella typhimurium*

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In this paper we demonstrate the existence of a second proline permease, gene *proP*, in *Salmonella typhimurium*. Uptake assays demonstrate that this second proline permease has 5 to 10% the uptake rate of the *putP* permease, the cell's major proline permease, when assayed at 20 μM proline. Genetic mapping by Hfr and P22-mediated genetic crosses placed the second proline permease gene at 92 min on the *S. typhimurium* genetic map, near the genes for melibiose utilization. F'-mediated complementation tests indicated that *Escherichia coli* also has the *proP* gene.

Multiple uptake systems for a single substrate have been demonstrated for sugars, amino acids, and inorganic ions (for reviews, see references 7, 19, 34). A proline auxotroph can be fed by low levels (16 μM) of exogenous proline provided that either of two proline uptake systems is active. A proline auxotroph with a defect in both uptake systems fails to be supplemented by such low levels of exogenous proline. One of these two transport systems is encoded by the *putP* gene, which has been previously mapped and described (23). This paper presents evidence for the second proline uptake system (*proP*).

MATERIALS AND METHODS

Media and growth conditions. Nutrient broth (0.8%) (Difco Laboratories) containing 0.5% NaCl was used as a complex medium. Vogel and Bonner E medium containing 2% glucose was used as minimal medium (32). Other carbon sources were used at 0.2% in the NCE medium of Berkowitz et al. (6). Medium in which proline is sole nitrogen source (PSN) has been previously described by Ratzkin and Roth (24). Except as noted, amino acids were added as needed at approximately 0.3 mM. Adenine was used at 0.4 mM as needed. Tetracycline was added to complex media at 25 μg/ml and to minimal medium at 10 μg/ml. Solid media contained 1.5% agar (Difco) except for PSN medium, which was solidified by 1.0% Ionagar (Oxoid). Cells were grown at 37°C unless otherwise indicated. Liquid cultures were grown with gyratory shaking.

Chemicals. The ^{3}H-labeled proline was purchased from Schwarz/Mann. Other chemicals used were of reagent grade or better and purchased from a variety of commercial sources.

Transductions. Bacteriophage P22 containing the mutation HT105/1 (29), which causes an increased frequency of generalized transduction, and a second mutation, *int-201* (3), which prevents prophage integration, was used for all transductions. Phage were grown on a donor strain by diluting a stationary culture of the donor 1:5 with nutrient broth containing 10^8 plaque-forming units of P22 HT *int* per ml and then growing the cells for 4 to 12 h at 37°C. Cells and debris were removed by centrifuging the culture at 5,000 rpm for 10 min in a clinical table-top centrifuge. The supernatant was placed over chloroform, blended in a Vortex mixer, and allowed to stand for 4 h before use. Approximately 50 μl of the supernatant was mixed with 100 μl of cells in transductions. In most crosses, phage and bacteria were mixed directly on selective media. When selection was made for inheritance of a donor Tn*10* element (Tet^r), phage and bacteria were mixed and preincubated for 30 min in a nonselective liquid medium before plating on tetracycline-containing solid media. Transductant clones were purified and made phage-free by streaking first on nutrient broth and then on selective medium.

Hfr crosses. In all Hfr crosses, the donor was diluted 1:10 into nutrient broth from an overnight stationary culture. After 2 h of growth at 37°C, 0.1 ml of this culture was used with 0.1 ml of a midlog recipient culture. This mixture was plated directly on selective media. The selected recombinants were picked and purified once selectively. These purified recombinants were picked to a master plate and replica printed to score coinheritance of unselected markers.

F' transfers. To transfer F' episomes, a 1:1 mix of donor and recipient was spotted onto selective medium. After 18 h of growth, several hundred colonies appeared in the mix whereas no colonies appeared in either donor or recipient controls. Exconjugant colonies were purified selectively.

To distinguish *melA* from *melB* Tn*10* insertion mutants, F_{tet14} *lac*$^+$ was transferred from TR2647 into the Mel$^-$ strains to be tested. Crosses were performed on minimal lactose medium. Purified Lac$^+$ exconjugants which became phenotypically Mel$^+$ were assigned the *melB* genotype since the *lac* permease (*lacY*) can transport melibiose, the substrate of the *melB* permease. Those Lac$^+$ exconjugants which remain phenotypically Mel$^-$ were assigned the *melA* genotype. All operations were carried out at 30°C.

Strains. The strains used for this study are derivatives of *Salmonella typhimurium* LT2 or LT7. Table 1 lists the numerical designations, full genotypes, and maternal origins (LT2 or LT7) of strains used in this

study. Unless otherwise indicated, the isolation or construction of all strains is described in this paper. Strains whose designation bears the prefix "TT" either contain a transposable drug resistance element (Tn*10*) or were derived from parental strains that contained such an element. The detailed nomenclature for Tn*10* insertion mutations has been described elsewhere (8, 15).

Uptake assays. Cells for uptake assays were grown to a density of approximately 10^8 cells/ml in minimal E medium supplemented with 2 mM proline at 37°C and then harvested by centrifugation and washed three times with buffer A, a synthetic phosphate-buffered medium (30). For uptake assays, cells were suspended to a concentration of between 100 and 400 μg of cell protein per ml in buffer A supplemented with 11 mM glucose. Cell protein was determined by the method of Lowry et al. (17), with bovine serum albumin as a standard. Before an assay, the cells (in buffer A plus glucose) were incubated for 5 min at 37°C. To initiate the assay, 400 μl of the cell suspension was added to the radioactive proline with the specific activities indicated in Table 5. Portions of 100 μl were filtered at 10-, 30-, and 50-s time intervals on cellulose-acetate cellulose-nitrate filters (0.45-μm pore size) with a vacuum. Immediately after filtering, the cells were rinsed with 10 ml of a buffer containing 0.01 M Tris-hydrochloride (pH 7.3), 0.15 M NaCl, and 0.5 mM $MgCl_2$. Filters were dried and counted in a toluene-based scintillation fluid as previously described (5). The amount of label taken up between the 10- and 30-s and the 30- and 50-s time intervals was used to calculate the uptake rate. If the two values differed by more than 20%, the results were disregarded and the assays were repeated. Errors of this type are most likely due to filtering inconsistencies. All assays were done in triplicate with the reproducibility indicated in Table 6.

RESULTS

Isolation of permease mutants. Starting with a *proAB47* deletion strain, Miklavz Grabnar obtained a derivative, TR1995, which failed to grow on low levels of exogenous proline (16 μM) but grew normally when supplemented with higher levels (2 to 10 mM) (23). The parental strain (*proAB47*) grows in response to the low (16 μM) level of exogenous proline. TR1995 was obtained after diethyl sulfate mutagenesis of *proAB47* and subsequent penicillin enrichment for mutants unable to grow on medium with the low proline concentration (16 μM). Evidence described below demonstrates that TR1995 carries two distinct mutations, each effecting an independent proline uptake system.

TR1995 has previously been shown to be unable to grow on proline as a sole nitrogen source (Put^-) (23). The Put^- phenotype is due to the genetic lesion *putP639*; this mutation affects the gene encoding the cells major proline permease (*putP*) (23, 24). When TR1995 is transduced to Put^+ (ability to grow on proline as sole nitrogen source) by using phage grown on wild-type cells, all the transductants regain the ability to satisfy their auxotrophic proline requirement with 16 μM exogenous proline. It would appear that a defect in the cell's *putP* gene results in the cell's inability to use low levels of exogenous proline. Subsequent strain construction proved that the situation was more complicated.

TABLE 1. *Strains used*

Strain designation	Genotype	Derivative of wild type
proAB47	*proAB47*	LT7
TR1995	*proAB47 putP639 proP673*	LT7
TR2433	HfrK6 *hisD8456 serA12*	LT2
TR2647	*pyrC7 strA1*/F'_{ta114}*lac*$^+$	LT2
TR4838	*purA115 hisC483 leuD798 fol-101*/F'_{117}*proP*$^+$	LT2
TR5279	*proAB47 putP639*	LT7
TR5280	*proAB47 proP673*	LT7
TR5281	*proAB47 putPA523*	LT2
TR5300	*proAB47 putP639 proP673*/F'_{117}*proP*$^+$	LT7
TT1667	*melB356*::Tn*10*	LT2
TT1672	*melA361*:Tn*10*	LT2
TT1798	*proAB47 putP639 zjd-27*::Tn*10*	LT7
TT1800	*proAB47 putP639 proP673 zjd-27*::Tn*10*	LT7
TT1801	*proAB47 putPA523 proP673 zjd-27*::Tn*10*	LT2
TT1803	HfrK6 *hisD845 serA12 zjd-27*::Tn*10*	LT2
TT1845	*metA53 purA115 pyrB655 rha-67*	LT2

Several other *putP* mutants were made Pro^- by introduction of a *proAB47* deletion; unlike TR1995, these Put^- strains were able to utilize low proline concentrations to satisfy their proline requirement. Apparently, TR1995 owes its uptake defect to the combination of *putP639* and a second mutation.

When TR1995 is transduced to growth on minimal medium with low (16 μM) proline by phage grown on *proAB47*, two types of transductants are seen. One type is the expected $putP^+$ transductant type. A second type is still Put^- and makes a small colony on nutrient broth plates. The second type of transductant has not received the donor's *putP* region; this can be demonstrated by transducing TR1995 to growth on 16 μM proline with phage grown on a donor strain deleted for all the *put* genes (TR5281: *putPA523 proAB47*). All transductants from this cross are Put^- and make small colonies on nutrient broth plates (Table 2). The results are best explained by postulating that TR1995 carries two mutations affecting proline transport, *putP* and a second mutation which we designate *proP*. When either of these mutations is corrected by transduction, the auxotrophic proline requirement may be supplied by low (16 μM) exogenous proline.

Isolation of a Tn*10* insertion near *proP*. To aid in the characterization of *proP*, we se-

TABLE 2. *Transductional crosses demonstrating the existence of the proP gene*[a]

Donor	Phenotype selected	Transductants observed
proAB47 (proP$^+$ *putP*$^+$*)*	Put$^+$	100/100 of the transductants were able to grow on 16 μM proline and formed large colonies on nutrient broth *(putP*$^+$ transduced in)
	Growth on 16 μM proline	39/100 of the transductants were Put$^+$ and formed large colonies on nutrient broth *(putP*$^+$ transduced in); 61/100 of the transductants were Put$^-$ and formed small colonies on nutrient broth *(proP*$^+$ transduced in)
TR5281 *(proAB47 proP*$^+$ *putPA523)*	Put$^+$	No transductants were obtained
	Growth on 16 μM proline	100/100 of the transductants were Put$^-$ and formed small colonies on nutrient broth *(proP*$^+$ transduced in)

[a] The recipient in all crosses was TR1995, whose genotype is inferred to be *proAB47 putP639 proP673*. Transductions were performed as described in Materials and Methods.

lected a Tn*10* insertion near the *proP* locus. To accomplish this, Tn*10* was allowed to insert randomly into the chromosome of *S. typhimurium* LT2 (14, 15). Approximately 3,000 independent insertion mutants were then grown in mixed culture. Phage P22 was grown on this pool, and the lysate was used to transduce TR1995 to growth on 16 μM proline. The transductants were screened for those which had received Tn*10* by cotransduction with the selected marker. proAB$^+$ and Put$^+$, as well as ProP$^+$, transductants could be identified which had coinherited Tn*10*. Several proP$^+$ Tet^r clones were purified, and linkage of Tn*10* to *proP* was tested in P22-mediated transductional crosses. One strain, TT1798 (*proAB47 putP639 proP*$^+$ *zjd-27*::Tn*10*), proved to have a Tn*10* insertion which was 70% linked to *proP*. This insertion (*zjd-27*::Tn*10*) is unlinked to the *put* region.

It was stated earlier that double mutants defective in both *proAB* and *putP* genes are able to grow on 16 μM proline. Our assumption was that a functional *proP* gene allowed growth on low levels of exogenous proline. The Tn*10* element inserted near *proP* permits us to test this hypothesis. Strain TT1800 (*proAB47 putP639 proP673 zjd-27*::Tn*10*) was constructed by transducing TR1995 to Tet^r with phage grown on TT1798 and identifying transductants unable to use the low proline concentration (16 μM). The phage grown on TT1800 was then used to transduce TR5281 (*proAB47 putPA523*) to Tet^r. Consistent with our hypothesis about *proP*, 70% of the Tet^r transductants were unable to use low proline. One such transductant was purified and designated as TT1801.

Hfr mapping. To facilitate genetic mapping of *proP*, we determined the chromosomal map position of the closely linked Tn*10* insertion whose phenotype (Tet^r) can be scored in any genetic background. The strain TR2433 (HfrK6 *hisD8456 serA12*) was transduced to Tet^r with phage grown on TT1798. The resultant strain, TT1803, retained the parental Hfr character. TT1803 was then used as a donor in conjugational crosses with a variety of auxotrophic strains. In all the crosses, prototrophy was selected (*his* and *ser* were counterselective markers), and the coinheritance of Tet^r was scored as described in Materials and Methods. The highest degree of linkage (75% coinheritance) was observed with the *purA* gene at 93 min on the *S. typhimurium* genetic map. To locate *zjd-27*::Tn*10* relative to *purA* and other markers in this region, the Hfr strain TT1803 was mated to a multiply marked strain carrying three mutations in the region of interest, *metA53*, *purA115*, and *pyrB655* (TT1845). Selection was made for PyrB$^+$ recombinants, which were purified and scored for coinheritance of PurA$^+$, MetA$^+$, and Tet^r. The results (Table 3 and Fig. 1) place *zjd-27*::Tn*10* between *metA* and *purA*.

In performing Hfr crosses with the Hfr containing Tn*10*, we encountered no difficulties provided Tet^r was scored and not selected. Several crosses were performed in which Tet^r was selected. These crosses gave anomalous results. The reason for this is not clear, but may involve the ability of Tn*10* to transpose (14).

To confirm our assignment of *zjd-27*::Tn*10* and the linked *proP* gene to the *purA* region of the chromosome, *Escherichia coli* episome F'_{117} (which includes this region) was transferred into

TABLE 3. *Hfr mapping of the Tn10 insertion mutation linked to proP*[a]

Phenotype of *pyrB*$^+$ recombinant	Regions of crossover events required for indicated recombinant	Total no. observed
PurA$^+$ Tet^r MetA$^+$	(1, 5)	65
PurA$^+$ Tet^r MetA$^-$	(1, 4)	19
PurA$^+$ Tet^s MetA$^+$	(1, 3, 4, 5)	0
PurA$^+$ Tet^s MetA$^-$	(1, 3)	6
PurA$^-$ Tet^r MetA$^+$	(1, 2, 3, 5)	0
PurA$^-$ Tet^r MetA$^-$	(1, 2, 3, 4)	2
PurA$^-$ Tet^s MetA$^+$	(1, 2, 4, 5)	0
PurA$^-$ Tet^s MetA$^-$	(1, 2)	1

[a] Results were obtained from the conjugation of TT1803 (HfrK6 *hisP843 serA12 zjd-27*::Tn*10*) with the recipient TT1845 (*metA53 purA115 pyrB65J rha-67*). PyrB$^+$ was selected and MetA$^+$, PurA$^+$, and Tet^r were scored. The regions of possible crossover events are diagramed in Fig. 1.

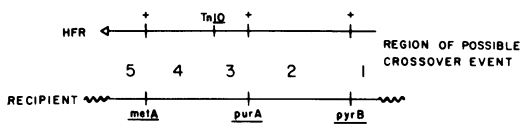

FIG. 1. *Hfr mapping of the Tn10 insertion mutation linked to proP showing map order as inferred from the data in Table 3.*

TR1995 (from the donor TR4838). The appearance of exconjugants able to grow on 16 μM proline implies that the F′$_{117}$ plasmid includes an *E. coli* version of the *proP* gene. A single exconjugant was purified and designated TR5300. To verify the episomal location of the *proP* gene in TR5300, cells were grown in acridine to induce episome segregation (11). The results (Table 4) show that the low proline growth phenotype in TR5300 was sensitive to acridine, whereas the ability of TR5279 (chromosomal *proP*$^+$) to grow on low proline was stable in the presence of acridine. These results indicate that the episome F′$_{117}$ includes the *proP* gene and that this gene must map in the chromosomal region including *pyrB* and *melB*.

Isolation and transduction of *melA* and *melB* Tn*10* insertions. An examination of the *S. typhimurium* genetic map (28) shows that the *melA-melB* gene cluster lies in a region near *purA* and hence is near *proP* as indicated by our mapping data. Data below demonstrate transductional linkage of *proP* to *mel*.

Several Mel mutants available to us proved to be too leaky for convenient testing of P22-mediated transductional linkage. To obtain nonleaky Mel mutants, Tn*10* was allowed to insert randomly into the chromosome of *S. typhimurium* LT2 (14, 15). These random insertions were then replica printed to eosin methylene blue-melibiose medium containing tetracycline at 25 μg/ml. Potential Mel$^-$ colonies were identified by their white appearance on the indicator plates. Such white colonies were purified and further characterized by examining growth on a variety of carbon sources. True Mel$^-$ clones, those unable to grow on melibiose but showing normal growth on other carbon sources tested, were designated as either *melA* or *melB*, depending on the ability of their Mel$^-$ phenotype to be complemented by an episomal *lacY*$^+$ gene. It has been demonstrated that *melB* (thiomethyl galactoside permease II) but not *melA* (α-galactosidase) can be complemented by *lacY*$^+$ (galactoside permease) (4, 20). The procedural details of the complementation tests are given in Materials and Methods. Tn*10* insertions designated as *melA* or *melB* are given in Table 1.

To examine transductional linkage of *proP* to the *melA-melB* gene cluster, the following crosses were performed. Phage P22 was grown on the *mel*::Tn*10* insertions given in Table 5. These phage were used to transduce TR1995 to Tet^r. All transductants became Mel$^-$, and approximately 20% of the transductants gained the ability to grow on 16 μM proline while remaining Put$^-$. Crosses performed selecting growth on 16 μM proline also showed coinheritance of *mel* and *proP*. These crosses (Table 5) indicate that *proP* is 20% linked to the *melA-melB* gene cluster by P22-mediated transductional crosses. This assignment is consistent with the F′-episome complementation and Hfr mapping.

TABLE 4. *Demonstration of a proP gene on the F′$_{117}$ episome*[a]

Strain	Fraction of clones showing growth on 16 μM proline after:	
	No acridine treatment	Acridine treatment
TR1995 (*proAB47 putP639 proP673*)	0/100	0/100
TR5279 (*proAB47 putP639 proP*$^+$)	100/100	100/100
TR5300 (*proAB47 putP639 proP673/ F′$_{117}$proP*$^+$)	100/100	0/100

[a] For growth with acridine, cells were diluted 10^{-5}-fold into fresh nutrient broth medium with acridine orange at 50 μg/ml. Cells were grown into stationary phase in the dark. Controls without acridine were treated identically. After growth in liquid, cells were plated for single colonies on rich medium. Single colonies were picked to a nonselective master plate which was replica plated to score ability to grow on low proline. (In this test, proline is used only to satisfy the strain's proline auxotrophy.)

Uptake assays. Discussion to this point has assumed that *proP* codes for a proline uptake system. To demonstrate this, proline uptake was measured in a variety of strains with various combinations of *proP* and *putP* mutations (Table 6). Uptake values for strains TR1995 and TT1801 demonstrate that the residual proline uptake activity in *putP* defective mutants (TR5279 and TR5281) is provided by the presence of a functional *proP* gene. Comparison of the uptake values of *putP* strains (TR5279 and TR5281) with that of a *proP* strain (TR5280) demonstrates that under the conditions used, *proP* gives the cell an uptake rate of approximately 5% that of the *putP* system in cells grown in minimal glucose medium supplemented with proline. The uptake rates in a *proP*$^+$ *putP*$^+$ strain (proAB47) and a *proP putP*$^+$ strain (TR5280) are approximately equal, a result consistent with *proP*'s contribution being a minor one under our assay conditions.

DISCUSSION

The transduction of TR1995 to growth on 16 μM proline demonstrates that either of two genes can allow a proline auxotroph to grow on low levels of exogenous proline. One of these genes is *putP*, which has previously been

TABLE 5. *Cotransduction of proP and mel*[a]

Donor	Phenotype selected	Transductants observed
TT1667 (*melB356*::Tn*10*)	Tet^r	11/50 Mel$^-$, able to grow on 16 μM proline (*proP*$^+$ cotransduced) 39/50 Mel$^-$, unable to grow on 16 μM proline
	Growth on 16 μM proline	38/100 Pro$^-$ Put$^+$ Tet^s Mel$^+$ (*putP*$^+$ transduced in) 49/100 Pro$^-$ Put$^-$ Tet^s Mel$^+$ (*proP*$^+$ transduced in) 5/100 Pro$^-$ Put$^-$ Tet^r Mel$^-$ (*proP*$^+$ transduced in, Tn*10* cotransduced) 8/100 Pro$^+$ Put$^-$ Tet^s Mel$^+$ (*proAB*$^+$ transduced in)
TT1672 (*melA361*::Tn*10*)	Tet^r	11/50 Mel$^-$, able to grow on 16 μM proline (*proP*$^+$ cotransduced) 39/50 Mel$^-$, unable to grow on 16 μM proline
	Growth on 16 μM proline	22/100 Pro$^-$ Put$^+$ Tet^s Mel$^+$ (*putP*$^+$ transduced in) 52/100 Pro$^-$ Put$^-$ Tet^s Mel$^+$ (*proP*$^+$ transduced in) 14/100 Pro$^-$ Put$^-$ Tet^r Mel$^-$ (*proP*$^+$ transduced in, Tn*10* cotransduced) 12/100 Pro$^+$ Put$^-$ Tet^s Mel$^+$ (*proAB*$^+$ transduced in)

[a] The recipient in all crosses was TR1995 (*proAB47 putP639 proP673 mel*$^+$). Transductions were performed as described in Materials and Methods.

TABLE 6. *Proline uptake assays*[a]

Strain	Permease alleles		Uptake rate (nmol/min per mg of cell protein) at:	
	putP	*proP*	2 μM proline	20 μM proline
proAB47	*putP*$^+$	*proP*$^+$	3.8, 3.3	6.5, 8.0
TR5280	*putP*$^+$	*proP673*	3.9, 2.4	8.5, 5.0
TR5279	*putP639*	*proP*$^+$	0.1, 0.04	0.4, 0.2
TR5281	*putPA523*	*proP*$^+$	0.1, 0.1	0.4, 0.4
TR1995	*putP639*	*proP673*	<0.02, <0.02	<0.02, <0.02
TT1801	*putPA523*	*proP673*	<0.02, <0.02	<0.02, <0.02

[a] Uptake determinations were made as described in Materials and Methods. The first value in each column were determined with proline at a specific activity of 500 Ci of ^{3}H per mol; the second values were made at a specific activity of 25 Ci of ^{3}H per mol. Determinations made for strains *proAB47* and TR5280 were reproducible within a ±15% error. Determinations made with strains TR5279 and TR5281 were reproducible within a ±50% error. The minimal uptake value judged significant is 0.02 nmol/min per mg of cell protein.

mapped at 22 min on the *S. typhimurium* genetic map. The *putP* gene encodes a major proline permease which is essential for the utilization of proline as a sole nitrogen source (23, 24). Through Hfr and phage P22-mediated genetic exchange the second gene, which we designated *proP*, has been shown to map at 92 min on the *S. typhimurium* genetic map, near the *melA-melB* gene cluster. Uptake assays demonstrate that *proP* codes for a second proline permease activity. In bacteria, multiple uptake routes for a single amino acid seem to be common. They have been demonstrated for histidine (1, 16), leucine (21, 22), arginine (25, 26), alanine (33), and aromatic amino acids (1, 2).

Uptake assays reported here were performed on cells grown in minimal glucose medium supplemented with proline. Under our conditions (20 μM proline), the *proP* system has approximately 5% of the uptake rate of the *putP* system. Proline has been shown to induce the *putP* system two- to threefold (24). The contribution of the *proP* system to cells grown without induction of the *putP* system may approach more nearly 10% of the total proline uptake activity at 20 μM proline. The previous failure to detect such an extent of residual proline uptake in *putP* mutants of *S. typhimurium* is probably due to differences in the methods used to measure uptake rates (23). Previous analysis of proline transport in membrane vesicles of *E. coli* is also likely to be a measure of the *putP* system (27, 31). We make these conclusions because the *putP* system clearly is the major route for proline transport.

Motojima et al. have recently reported the isolation of mutants which fail to have their auxotrophic proline requirement (due to a *proA* mutation) supplemented by 43 μM proline but which grow normally in the presence of 3.5 mM proline (18). These workers have attributed the defect in proline uptake to a single locus, *proT*, which they map at 82 min on the *E. coli* genetic map. Uptake assays demonstrate that the *proT* strains have only 1.5 to 3.0% the transport activity of the parental *proT*$^+$ strain. Our observation that *putP* (22 min on the *S. typhimurium* genetic map) is a major proline transport gene is difficult to reconcile with an essentially complete loss of transport activity due to a *proT* mutation at 82 min on the *E. coli* genetic map. The report of *put* mutants at an identical map position (22 min and linked to *pyrC*) in *E. coli* K-12 by Condamine (9) implies that differences between *E. coli* and *Salmonella* do not account for the discrepancy. Ratzkin and Roth have analyzed over 100 mutants defective in proline utilization and found that all map at 22 min on the *S. typhimurium* genetic map (24). This observation suggests that all components of the *putP* permease system map at the *put* gene cluster. The *proP* mutation we have described arose as one of two mutations present in a strain selected by a scheme very similar to that of Motojima et al. (18). Before interpreting the role of the proposed *proT* gene, rigorous genetic analysis is required to rule out the possibility that the *proT* strains might owe their phenotype to multiple mutations. It is important to know whether the *proT* strains are able to use proline as a sole nitrogen source. If the *proT* strains are phenotypically Put$^-$, the map position of the *put* mutation in the *proT* strains may help resolve some of the discrepancies between our results and those of Motojima et al.

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