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A Third L-Proline Permease in *Salmonella typhimurium* Which Functions in Media of Elevated Osmotic Strength

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Exogenous proline specifically stimulates the growth rate of enteric bacteria in media of inhibitory osmotic strength (J. H. B. Christian, Aust. J. Biol. Sci. 8:490–497, 1955). I observed that *Salmonella typhimurium* mutants which lack both of the previously known proline permeases (*putP proP*) are stimulated by proline in media of inhibitory osmolarity. I propose that there is a third proline permease which functions only in media of elevated osmolarity. This conclusion is based on the observations that, in media of elevated osmolarity, (i) the sensitivity of *putP proP* mutants to toxic proline analogs increases, (ii) proline requirements for maximal growth of proline auxotrophic *putP proP* mutants decreases, and (iii) the specific rate of incorporation of radioactive proline into protein of growing cells increases. I obtained a Tn*10*-induced mutation in a gene (*proU*) required for the functioning of the third proline permease and determined the map location to be at 59 map units of the chromosome, between *srlA* and *tct*, 66% linked to *nalB* in P22 transduction. My results suggest that the function of the third, osmotically stimulated permease might be to accumulate high intracellular proline levels during osmotic stress. Possible mechanisms by which proline might cause growth stimulation are discussed.

In *Salmonella typhimurium* and *Escherichia coli*, two transport systems for L-proline have been described. The first proline permease (PP-I) is required for the transport of proline when that compound is the sole carbon or nitrogen source (14, 15, 22). PP-I is induced by proline, and it is subject to control by catabolite repression. There is a second proline permease (PP-II) whose expression has been shown to be elevated in response to amino acid starvation in *S. typhimurium* (1). Mutations, at a locus called *putP*, which inactivate PP-I have been extensively studied in both *S. typhimurium* and *E. coli* (14, 15, 22), but only one mutation inactivating PP-II, which is at the *proP* locus in *S. typhimurium*, has been described (1, 12, 14).

In addition to serving as a carbon or nitrogen source and as a constituent of proteins, proline has an interesting third function in microbial physiology: it stimulates the growth rate of some microorganisms in media of elevated osmotic strength. This phenomenon was discovered by Christian, using *Salmonella oranienburg* (3, 4), and it has been demonstrated in other enteric bacteria (6; D. Le Rudulier et al., manuscript in preparation). I have extensively characterized this effect in *S. typhimurium* and used it as the rationale for the isolation of mutations that result simultaneously in proline overproduction and increased osmotolerance in that species (6) and in related enteric organisms (Le Rudulier et al., in preparation).

The work presented here was carried out to determine which of the proline permeases of *S. typhimurium* mediates the stimulatory effect of proline under conditions of osmotic inhibition. I observed that proline was stimulatory to mutants lacking both PP-I and PP-II (*putP proP*). I present evidence that this residual stimulation is due to a third proline permease (PP-III) which functions only in media of elevated osmolarity, and I describe some characteristics of mutant strains lacking this third proline permease.

MATERIALS AND METHODS

Media and growth conditions. The rich medium used was LB (8). The minimal medium used was medium 63 (5), consisting of 0.1 M KH_2PO_4, 0.075 M KOH, 0.015 M $(NH_4)_2SO_4$, 0.16 mM $MgSO_4 \cdot 7H_2O$, and 1.8 μM $FeSO_4 \cdot 7H_2O$. Medium of elevated osmotic strength was obtained by the addition of NaCl or other solutes at concentrations as indicated; in the case of NaCl, it was necessary to readjust the pH to that of medium 63 (pH 7.2), which was done by the addition of NaOH. Unless otherwise indicated, 10 mM glucose was the

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carbon source. When used, melibiose was at 5 mM. When proline was used as the nitrogen source, the $(NH_4)_2SO_4$ in medium 63 was replaced with 0.015 M K_2SO_4, and proline was added at 5 mM with 20 mM sodium succinate as the carbon source. When the Put$^+$ phenotype was selected in transductions, nutrient broth (Difco Laboratories) was included in this ammonium-free medium at a final concentration of 0.3 g/liter (15). For the growth of proline auxotrophs possessing PP-I (*putP*$^+$) or PP-II (*proP*$^+$), a proline supplement was included in minimal medium at a 0.2 mM concentration, and for the growth of proline auxotrophs lacking both PP-I and PP-II (*putP proP*), a proline supplement, at 1 mM, was included in both the minimal and the rich media. Kanamycin sulfate, when used, was at a concentration of 75 mg/liter, and tetracycline was used at 10 mg/liter in minimal medium and at 15 mg/liter in rich medium. Nalidixic acid sodium salt, when used, was at a concentration of 4 mg/liter in LB plates (19). Solid media contained an additional 20 g of agar (Difco) per liter. Unless otherwise indicated, cultures were grown aerobically at 37°C.

Bacterial strains. *S. typhimurium* strain TL1 (wild-type strain LT2) was obtained from J. L. Ingraham. *S. typhimurium* LT2 strains TR1995 [Δ(*proBA*)*47 putP639 proP673*] (14), TT1672 (*melA361*::Tn*10*) (12), TT1801 [Δ(*proBA*)*47* Δ(*putPA*)*557 proP673 zjd-27*::Tn*10*] (12), TT2601 (*putA842*::Tn*5*), and TT2660 [Δ(*putPA*)*557 zcc-628*::Tn*5*] were from J. R. Roth. Strain KS296 (*nalB*) (19), used to map the *proU* mutation (see below), was from J. Somers. The construction of strains TL106 (*putA842*::Tn*5*) and TL117 [Δ(*proBA*)*47*] has been described previously (6).

The derivation of other strains used in this work is summarized in Table 1. All strains having the designation "TL" used here have been derived by transductions, Tn*10* transposition, or spontaneous mutations to proline analog resistance (see below) from strain TL1. To avoid complications arising from possible differential turnover of proline, or of proline analogs in various proline transport mutants (22), all experiments except one (see Fig. 5A) were carried out with proline dehydrogenase, Δ^1-pyrroline-5-carboxylate dehydrogenase mutants (*putA*), which in the case of *putP*$^+$ strains carried the *putA842*::Tn*5* insertion and in the case of *putP* strains carried the *putPA-557* deletion.

Selection of *proP* mutants. Strains lacking PP-I (*putP*) are resistant to the toxic proline analog L-azetidine-2-carboxylate (Azt) (14, 22). I found that strains proficient in PP-II (*proP*$^+$) which carried *putPA* deletions were sensitive to another proline analog, 3,4-dehydro-DL-proline (Dhp), but derivatives of *putPA* strains which carried the *proP673* mutation were resistant (7). Therefore, additional *proP* mutants were obtained by spreading ~2 × 10^8 cells of strain TL135 [Δ(*putPA*)*557 proP*$^+$] on glucose minimal medium plates containing 0.3 mM Dhp. Resistant mutants appeared at the approximate frequency of one per 10^5 cells plated. Some of these mutants were characterized to carry *proP* mutations (see below).

Isolation of a Tn*10* insertion inactivating PP-III. A Tn*10*-induced mutation inactivating the *proU*$^+$ gene, which is defined here as a gene required for PP-III, was obtained in the following manner. Strain TL177 (*putP proP*) was infected with a heat-induced P22 lysate of strain NK337 [*hisC527 leu-414 supE* (P22 *C2ts29 12amN11 13amH101 int-3* Tn*10*)] prepared by L. S. Pierson III as described by Davis et al. (8). Approximately 3 × 10^9 cells in 1 ml were added to 1 ml of the undiluted phage lysate. The phage were allowed to adsorb in an unshaken culture at 37°C, and 0.1-ml aliquots were spread on 10 minimal plates containing 1 mM Azt, 0.3 M NaCl, and tetracycline and incubated at 42°C. Three colonies resistant to both tetracycline and Azt appeared. To determine whether the Tn*10* insertion inactivated the third proline permease, phage P22 was grown on the three strains, and the lysate was used to transduce the *putP proP* strains TL177 and TL179 to tetracycline resistance on LB plates. Ten tetracycline-resistant progeny obtained from each of the crosses were tested for resistance to 1 mM Azt in minimal medium containing 0.3 M NaCl. Of the three strains used as donors, one, TL187, gave rise to tetracycline-resistant transductants which proved to be resistant to Azt with 0.3 M NaCl. Thus, in that strain, the Tn*10* insertion caused loss of PP-III. The mutation was called *proU1655*::Tn*10*.

Transductions. Phage P22 HT105/1 *int-201*, obtained from J. R. Roth, was used in generalized transductions. The transductions were carried out as in reference 8.

Incorporation of [^{3}H]proline and [^{14}C]leucine into proteins. Exponentially growing cells were inoculated at a final density of 10^7 cells per ml into minimal medium containing 0.05 mM L-[^{3}H]proline (~10^{13} cpm/mol), 0.5 mM L-[^{14}C]leucine (~2 × 10^{11} cpm/mol) and, when used, 0.3 M NaCl, and the cultures were grown at 37°C. At various times, 0.1-ml samples were removed and pipetted into 1 ml of trichloroacetic acid (50 g/liter) and heated at 95°C for 15 min. These samples were filtered through GF/C fiber glass filters (Millipore Corp.), and the original test tubes and filters were washed with three portions of 4 ml of trichloroacetic acid, twice with 5 ml of distilled water, and twice with 5 ml of 95% ethanol. The filters were dried at 50°C and added to 5 ml of Aquasol (New England Nuclear Corp.), and their radioactive content was counted. The results are expressed as the number of proline molecules per leucine molecules incorporated into hot trichloroacetic acid-insoluble material (i.e., protein).

Chemicals. Azt and Dhp were from Calbiochem. Nalidixic acid sodium salt was from Sigma Chemical Co. L-[2,3-^{3}H]proline and L-[*U*^{14}-C]leucine were purchased from New England Nuclear Corp.

RESULTS

Isolation of *proP* mutations. The idea that there might be a third proline permease in *S. typhimurium* was initially prompted by the observation that the growth rate of *putP proP* double mutants was stimulated by proline in media of inhibitory osmolarity. Since the *proP673* allele was the only known mutation affecting *proP* (1, 12, 14), it was important to show that residual proline permease activity in *putP proP673* strains was not due to *proP673* being an atypical mutation which might be leaky or osmoremedial. Therefore, other *proP* mutations were isolated. Their selection was based on the observation that *putPA proP*$^+$ strains (which are

TABLE 1. List of strains constructed for this work

Strain	Genotype[a]					Derivation[b]
	putPA	*proP*	*proU*	*proBA*	Others	
TL131	*P*$^+$*A824*::Tn*5*	+	+	Δ(*proBA*)*47*		P22.TT2601→TL117 = Kan^r Put
TL135	Δ(*putPA*)*557*	+	+	+	*zcc-628*::Tn*5*	P22.TT2600→TL1 = Kan^r Put
TL137	Δ(*putPA*)*557*	+	+	Δ(*proBA*)*47*		P22.TT2600→TL117 = Kan^r Put
TL139	Δ(*putPA*)*557*	*proP673*	+	Δ(*proBA*)*47*	*zcc-628*::Tn*5* *zjd-27*::Tn*10*	P22.TT1801→TL137 = Tet^r, unable to grow with 0.016 mM proline
TL141	+	*proP673*	+	Δ(*proBA*)*47*	*zcc-628*::Tn*5* *zjd-27*::Tn*10*	P22.TL1→TL139 = Put$^+$ Kan^r
TL143	+	*proP673*	+	+	*zcc-628*::Tn*5* *zjd-27*::Tn*10*	P22.TL1→TL141 = ProB$^+$A$^+$
TL145	Δ(*putPA*)*557*	*proP673*	+	+	*zcc-628*::Tn*5* *zjd-27*::Tn*10*	P22.TL1→TL139 = ProB$^+$A$^+$
TL167	Δ(*putPA*)*557*	+	+	+	*melA361*::Tn*10* *zcc-628*::Tn*5*	P22.TT1672→TL135 = Tet^r
TL174	Δ(*putPA*)*557*	+	+	Δ(*proBA*)*47*	*melA361*::Tn*10* *zcc-628*::Tn*5*	P22.TT1672→TL137 = Tet^r
TL176	Δ(*putPA*)*557*	*proP1651*	+	+	*zcc-628*::Tn*5*	Spontaneous Dhp^r mutant of TL135; see text
TL177	Δ(*putPA*)*557*	*proP1652*	+	+	*zcc-628*::Tn*5*	Spontaneous Dhp^r mutant of TL135; see text
TL178	Δ(*putPA*)*557*	*proP1653*	+	+	*zcc-628*::Tn*5*	Spontaneous Dhp^r mutant of TL135; see text
TL179	Δ(*putPA*)*557*	*proP1654*	+	+	*zcc-628*::Tn*5*	Spontaneous Dhp^r mutant of TL135; see text
TL180	Δ(*putPA*)*557*	*proP673*	+	+	*zcc-628*::Tn*5*	P22.TR1995→TL167 = Mel$^+$ Dhp^r
TL182	Δ(*putPA*)*557*	*proP1651*	+	Δ(*proBA*)*47*	*zcc-628*::Tn*5*	P22.TL176→TL174 = Mel$^+$, unable to grow with 0.016 mM proline
TL183	Δ(*putPA*)*557*	*proP1652*	+	Δ(*proBA*)*47*	*zcc-628*::Tn*5*	P22.TL177→TL174 = Mel$^+$, unable to grow with 0.016 mM proline
TL184	Δ(*putPA*)*557*	*proP1653*	+	Δ(*proBA*)*47*	*zcc-628*::Tn*5*	P22.TL178→TL174 = Mel$^+$, unable to grow with 0.016 mM proline
TL185	Δ(*putPA*)*557*	*proP1654*	+	Δ(*proBA*)*47*	*zcc-628*::Tn*5*	P22.TL179→TL174 = Mel$^+$, unable to grow with 0.016 mM proline
TL186	Δ(*putPA*)*557*	*proP673*	+	Δ(*proBA*)*47*	*zcc-628*::Tn*5*	P22.TR1995→TL174 = Mel$^+$, unable to grow with 0.016 mM proline
TL187	Δ(*putPA*)*557*	*proP1652*	*proU1655*::Tn*10*	+	*zcc-628*::Tn*5*	Tn*10* insertion into *proU*, derivative of TL177 resistant to Azt with 0.3 M NaCl; see text
TL192	Δ(*putPA*)*557*	+	*proU1655*::Tn*10*	+	*zcc-628*::Tn*5*	P22.TL187→TL135 = Tet^r
TL195	Δ(*putPA*)*557*	*proP1654*	*proU1655*::Tn*10*	+	*zcc-628*::Tn*5*	P22.TL187→TL179 = Tet^r
TL196	Δ(*putPA*)*557*	*proP1654*	*proU1655*::Tn*10*	Δ(*putBA*)*47*	*zcc-628*::Tn*5*	P22.TL187→TL185 = Tet^r
TL197	+	*proP1654*	+	+		P22.TL1→TL179 = Put$^+$ Kan^s
TL198	*P*$^+$*A842*::Tn*5*	*proP1654*	+	+		P22.TT2601→TL197 = Kan^r Put
TL199	*P*$^+$*A842*::Tn*5*	*proP1654*	*proU1655*::Tn*10*	+		P22.TL187→TL198 = Tet^r

[a] The symbol *proU* refers to a gene defined in this publication. All other genetic symbols are defined in reference 16.

[b] The phenotypic designation Mel$^+$ denotes the ability to grow on melibiose. Put$^+$/Put refer to the ability/inability to use proline as a nitrogen source. ProB$^+$A$^+$ denotes proficiency in proline biosynthesis. Kan^r/Kan^s, Tet^r/Tet^s, Azt^r/Azt^s, and Dhp^r/Dhp^s denote resistance/sensitivity to 75 mg of kanamycin sulfate per liter, 10 to 15 mg of tetracycline per liter, 1 mM Azt, and 0.3 mM Dhp, respectively. The symbol P22.strain X→strain Y = phenotypes A, B, and C denotes that P22 lysate from strain X was used to transduce strain Y, selecting and screening phenotypes A, B, and C.

resistant to the proline analogue Azt [14]) are sensitive to the proline analogue Dhp, but *putPA proP673* derivatives are resistant to both compounds (7). Thus, additional *proP* mutations could be generated by selecting derivatives of *putPA* strains which were resistant to Dhp (see above). Four mutants, TL176, TL177, TL178, and TL179, isolated independently in this manner, were used for further analysis.

The *proP673* allele was shown to be linked to *melA* by P22 transduction (12). To check whether the mutations in the above strains giving rise to Dhp^r were in *proP*, I determined whether they were cotransducible with *melA*. The mapping was carried out by transducing strain TL174 (*melA*::Tn*10 putPA proBA*) to growth on melibiose minimal medium containing 1 mM proline, using P22 lysates obtained on the presumed *proP* strains. The inheritance of *proP* mutations was scored on the basis of the phenotype that proline auxotrophic strains which lack both PP-I and PP-II require high (>0.5 mM) proline for maximal growth rate, whereas strains which possess PP-II (e.g., TL174) can grow with a maximal growth rate at low (0.016 mM) proline (12, 14). Thus, 50 transductants, obtained by using strains TL176, TL177, TL178, and TL179 as donors, were tested for their ability to grow with 0.016 mM proline, and 52, 36, 48, and 32% of the transductants, respectively, were unable to do so. In the control experiment, with TR1995 (*putP proP673*) as the donor, 37% of the transductants could not grow with 0.016 mM proline. Therefore, on the basis of similar phenotypes conferred by *proP673* (resistance to Dhp in a *putP* genetic background and inability to grow with 0.016 mM proline in a *putP proBA* background), and on the basis of similar linkage to *melA*, I conclude that the mutations giving rise to Dhp^r in strains TL176, TL177, TL178, and TL179 are in the *proP* gene.

Proline transport defect in *putP proP* mutants overcome by growth in media of elevated osmolarity. The first test to determine whether there is a third proline permease which functions under conditions of osmotic inhibition was to determine whether *putP proP* mutants became sensitive to Azt or Dhp in media of elevated osmolarity. Strain TL179 (*putP proP*), which was resistant to Azt and Dhp in minimal medium (as compared with the *putP proP*$^+$ parental strain TL135), acquired sensitivity to both proline analogs in the presence of an additional 0.3 M NaCl (Table 2; see also Fig. 2). The *putP proP*$^+$ strain TL135 which, like TL179, is resistant to Azt in minimal medium, also becomes sensitive to it in the presence of 0.3 M NaCl. Restoration of Dhp or Azt sensitivity in the presence of 0.3 M NaCl was observed with over 20 independent *putP proP* strains, including some which carried *proP*

TABLE 2. Osmotic stress-induced increase in the proline analogue sensitivities of *putP proP*$^+$ or *putP proP* strains

Strain	Proline analog	Growth rate (generation per h)	
		Minimal medium	Minimal medium plus 0.3 M NaCl
TL106 (*putP*$^+$ *proP*$^+$)	None	0.98	0.65
	Azt	<0.05	<0.05
	Dhp	<0.09	<0.06
TL135 (*putP proP*$^+$)	Azt	0.86	<0.06
	Dhp	<0.12	<0.05
TL179 (*putP proP*)	Azt	0.87	0.18
	Dhp	0.81	<0.06

mutations caused by chromosomal rearrangements generated by *melA*::Tn*10* insertions (data not shown). Therefore, it seemed unlikely that this phenotype was a consequence of incomplete loss of *proP* function; the more plausible explanation seemed to be that there is a third proline permease.

As discussed above, proline auxotrophic strains which lack PP-I and PP-II (i.e., *proBA putP proP*) grow slowly or not at all with ≤0.1 mM proline. If there is a third proline permease in cells grown in media of elevated osmolarity, one might expect that the growth rate of such strains in the presence of low concentrations of proline would be stimulated by increasing the osmolarity of the medium. Such is the case (Table 3). The growth rates of five independently isolated *proP putP proBA* strains were about ninefold less than those of the *proP*$^+$ control strains TL131 (*putP*$^+$ *proBA*) and TL137 (*putP proBA*) in minimal medium with 0.1 mM proline. However, increasing the osmolarity of the medium stimulated the growth rates of the *proP putP proBA* strains so that, in the presence of 0.3 M NaCl, their growth rates were similar to those of strains TL131 and TL137. At proline concentrations > 0.2 mM, strain TL185 grew faster in the presence of 0.3 M NaCl than in minimal medium (Fig. 1). The dependence of the growth rate on proline concentration in the presence of 0.3 M NaCl is such that approximately 0.02 mM proline sustains half maximal growth rate of *proP putP proBA* strains. In minimal medium, 0.2 mM proline is required for half maximal growth rate.

The effects of other solutes on the growth rate of strain TL185 (*proP putP proBA*) with 0.1 mM proline were tested. The result was that 0.25 M K_2SO_4 or 0.44 M sucrose, which have approximately the same osmotic strength as 0.3 M NaCl (20), caused similar stimulation of the growth

TABLE 3. Stimulation of growth rate of *putP proP* proline auxotrophs by elevated osmolarity in the presence of limiting proline

Strain[a]	Proline (mM)	Growth rate (generation per h)	
		Minimal medium	Minimal medium plus 0.3 M NaCl
TL131 (*proBA putP*$^+$ *proP*$^+$)	None	<0.07	<0.06
	0.1	1.07	0.67
TL137 (*proBA putP proP*$^+$)	0.1	0.89	0.62
TL185 (*proBA putP proP1654*)	None	<0.05	<0.05
	0.1	0.10	0.59
TL182 (*proBA putP proP1651*)	0.1	0.10	0.62
TL183 (*proBA putP proP1652*)	0.1	0.11	0.57
TL184 (*proBA putP proP1653*)	0.1	0.09	0.60
TL186 (*proBA putP proP673*)	0.1	0.10	0.61

[a] The *proBA* in the strains in this table is Δ(*proBA*)*47*, and the *putP* mutation is Δ(*putPA*)*557*.

rate. Thus, the effect is not dependent on any specific solutes, be they electrolytes or nonelectrolytes. However, no stimulation was observed with 0.51 M glycerol (whose osmolarity also equals that of 0.3 M NaCl). Glycerol is known to be freely permeable through the cell membrane (9, 11) and, unlike the other solutes, it does not set up an osmotic gradient across the membrane. These observations suggest that proline transport into the cells is stimulated as a consequence of elevated external osmolarity, rather than as a consequence of the presence of any given solute. This increased proline transport might be due, as I suggested, to the functioning of a third proline permease (PP-III). Alternatively, it might be the result of the stimulation of a nonspecific leakage of proline across the membrane. To negate this second possibility, I isolated mutants lacking the osmotically activated proline permease.

Mutants lacking PP-III. A facile selection procedure for mutants lacking the third proline permease was afforded by the sensitivity of *putP proP* mutants to Azt or Dhp in media of elevated osmolarity. Starting with strains TL176 through TL180, over 50 independent spontaneous mutants were obtained which were resistant to 0.3 mM Dhp or 1 mM Azt in minimal medium containing 0.3 M NaCl. Also, a Tn*10*-induced mutation giving rise to the same phenotype was obtained (see above). The mutation was called *proU1655*::Tn*10*, with the corresponding wild-type allele being *proU*$^+$. Since the phenotype of *proU1655*::Tn*10* strains is very similar to isogenic strains carrying spontaneous mutations inactivating PP-III (data not shown), strains carrying *proU1655*::Tn*10* are used to illustrate the characteristics of *proU* mutants.

Phenotype of *proU* mutants. The effect of the *proU1655*::Tn*10* mutation on sensitivity to proline analogs is shown in Fig. 2. The *putP proP proU*$^+$ strain TL179, which is resistant to Azt and Dhp in minimal medium, was sensitive to both compounds with 0.3 M NaCl (Fig. 2A). However, the *proU*::Tn*10* derivative, TL195, was resistant to both proline analogs, even in the presence of 0.3 M NaCl (Fig. 2B). An interesting observation was made with the *putP proP*$^+$ *proU*::Tn*10* strain, TL192: this strain was resistant to Azt in minimal medium but sensitive to it in the presence of 0.3 M NaCl (data not shown). This phenomenon was not studied further, but it might indicate that Azt, which in minimal medium is a poor substrate for PP-II (1, 22), is taken up more rapidly by that permease in media of elevated osmolarity.

The *proU1655*::Tn*10* mutation was also introduced into proline auxotrophs which lacked PP-I or PP-II or both, and the proline requirements of these strains were investigated. As was seen earlier, 0.1 mM proline in minimal medium was insufficient to support optimal growth rate of *putP proP proU*$^+$ *proBA* strains, but increasing the osmolarity of the medium stimulated the growth rates of such strains (Fig. 3A). This stimulation was blocked upon the introduction of the *proU1655*::Tn*10* mutation into *putP proP proBA* strains (Fig. 3B). However, both *proU*$^+$ *putP proP proBA* and *proU putP proP proBA*

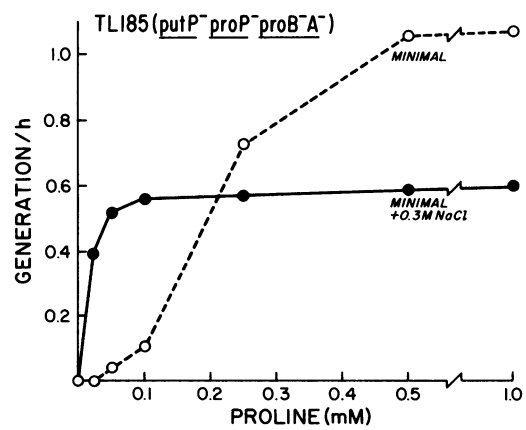

FIG. 1. The dependence of the growth rate of *putP proP proBA* strain TL185 on the exogenous proline concentration. Symbols: ○, TL185 grown in minimal medium; ●, TL185 grown in minimal medium plus 0.3 M NaCl.

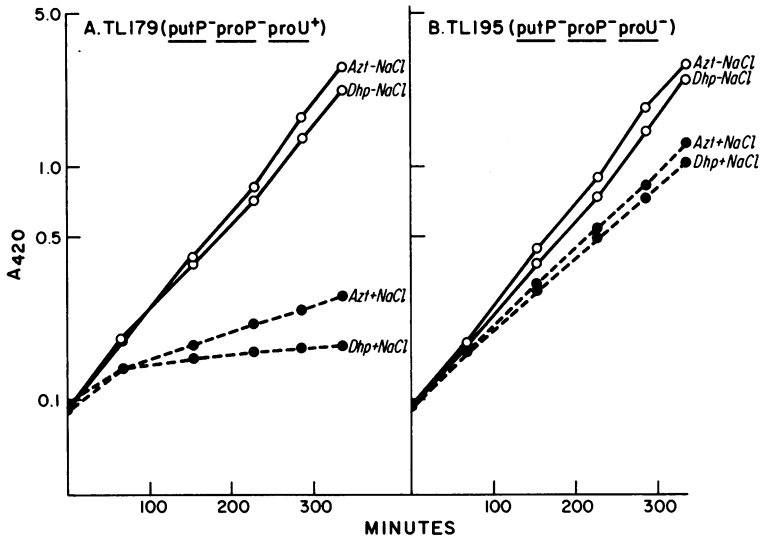

FIG. 2. The effect of *proU* mutation on the proline analog sensitivity of *putP proP* strains in the presence of 0.3 M NaCl. The optical density at 420 nm of cultures is plotted as a function of incubation time. Symbols: ○, growth in minimal medium; ●, growth in minimal medium plus 0.3 M NaCl, in each case with 1 mM Azt or 0.3 mM Dhp, as indicated. (A) Strain TL179 (*putP proP proU*$^+$); (B) strain TL195 (*putP proP proU*).

strains grew at nearly optimal growth rates (~1 generation per h) in minimal medium with 1 mM proline (Fig. 3). This indicates that proline at high external concentrations can still penetrate the cell membrane, either by some unknown carrier or by passive diffusion.

Proline uptake studies. I attempted to confirm directly that a proline permease is switched on in *putP proP* strains under conditions of osmotic inhibition by measuring the rate of uptake of radioactive proline by nongrowing cells. However, I was unable to obtain reliable results with the amino acid transport assay protocols of Anderson et al. (1) or Weaver and Konisky (21), using *putP proP* strains grown with 0.3 M NaCl. Therefore, I carried out a more qualitative experiment to demonstrate the residual proline transport ability of such strains: the incorporation of radioactive proline into protein of growing cells. The experiment consisted of growing cells in the presence of [^{3}H]proline (0.05 mM) and [^{14}C]leucine (0.5 mM) and determining the number of the proline and leucine molecules incorporated into protein at various times (see above). The specific rate of leucine incorporation (i.e., the number of leucine molecules incorporated per cell) was not influenced either by the presence or absence of 0.3 M NaCl or by the proline transport genotype of the strains (data not shown). Therefore, to minimize experimental error, the results are expressed as the ratio of proline molecules incorporated per leucine molecules incorporated. The incorporation of proline (per leucine molecules) was stimulated three- to sevenfold in the *putP proP*$^+$ *proU*$^+$ strain TL135 (Fig. 4A) or in the *putP proP*$^+$ *proU* strain TL192 (Fig. 4B) as a consequence of growth in 0.3 M NaCl. In the *putP proP proU*$^+$ strain TL179 proline incorporation in minimal medium was reduced about 10-fold from that seen with strain TL135, but in the presence of 0.3 M NaCl, proline incorporation was stimulated about 30-fold, so that under these conditions proline incorporation in strain TL179 (Fig. 4C) was similar to that seen with strain TL135. In the *putP proP proU* strain TL195 in minimal medium, the ratio of proline to leucine incorporation was even lower than that seen with the *putP proP proU*$^+$ strain TL179, and it was only very slightly stimulated by osmotic stress (Fig. 4D). These radioactive proline incorporation experiments are consistent with the conclusion that there is an osmotically stimulated proline permease in *putP proP proU*$^+$ strains which is damaged by the *proU1655*::Tn*10* mutation. These labeling experiments were carried out with proline prototrophs, and the rate of incorporation of proline into proteins may not be an accurate measure of proline transport since the rate of biosynthesis of proline, and hence the specific activity of the free intracellular proline pool, might be influenced by the NaCl concentration of the medium or by the proline transport genotype of the cells.

PP-II and PP-III mediation of the growth rate stimulation by proline during osmotic stress. The

observations presented here were based on an experiment designed to determine which of the previously known proline permeases were required to mediate the stimulation of growth rate of *S. typhimurium* by proline under conditions of osmotic stress. My initial finding is shown in Fig. 5A: the *putP* gene product was not required for the effect, but if the *proP* gene was inactivated, there was approximately a twofold reduction in the ability of proline to stimulate growth rate in minimal medium supplemented with 0.65 M NaCl. However, a residual stimulation was observed in *putP proP* strains. This residual stimulation was not specific to the *proP673* allele used in the experiment in Fig. 5A; it was observed with *proP1654* (Fig. 5B) and in over 20 other independently isolated *proP* mutants (data not shown).

Proline was not stimulatory at all to the *putP proP proU*::Tn*10* strain TL195 in minimal medium containing 0.65 M NaCl (Fig. 5B), implying that the residual stimulation of *putP proP proU*$^+$ strains was due to the *proU*$^+$ gene product. However, the *putP proP*$^+$ *proU*::Tn*10* strain TL192 was stimulated (even to a greater extent than the isogenic *putP proP proU*$^+$ strain TL179), indicating that the *proP* product can also mediate the stimulatory effect of proline. The stimulatory effect was absent in the *putP*$^+$ *proP proU* strain TL199, so that PP-I (which is necessary for growth with proline as the sole carbon or nitrogen source) did not mediate the stimulation of growth rate by proline in media of elevated osmolarity.

Chromosomal location of *proU*. The *proU1655*::Tn*10* mutation was mapped by the technique of Tn*10*-directed integration of F′$_{ts}$::Tn*10* *lac* and subsequent mobilization of the chromosome to determine origin of transfer (8). The initial observations were that *proU1655*::Tn*10* was between *cysC* and *cysA*, and these results were further refined so that *proU1655*::Tn*10* was located between *srl* and *tct*, which are at 58 to 60 map units of the *Salmonella* genetic map (16, 19). P22-mediated transduction revealed no linkage (<1%) between

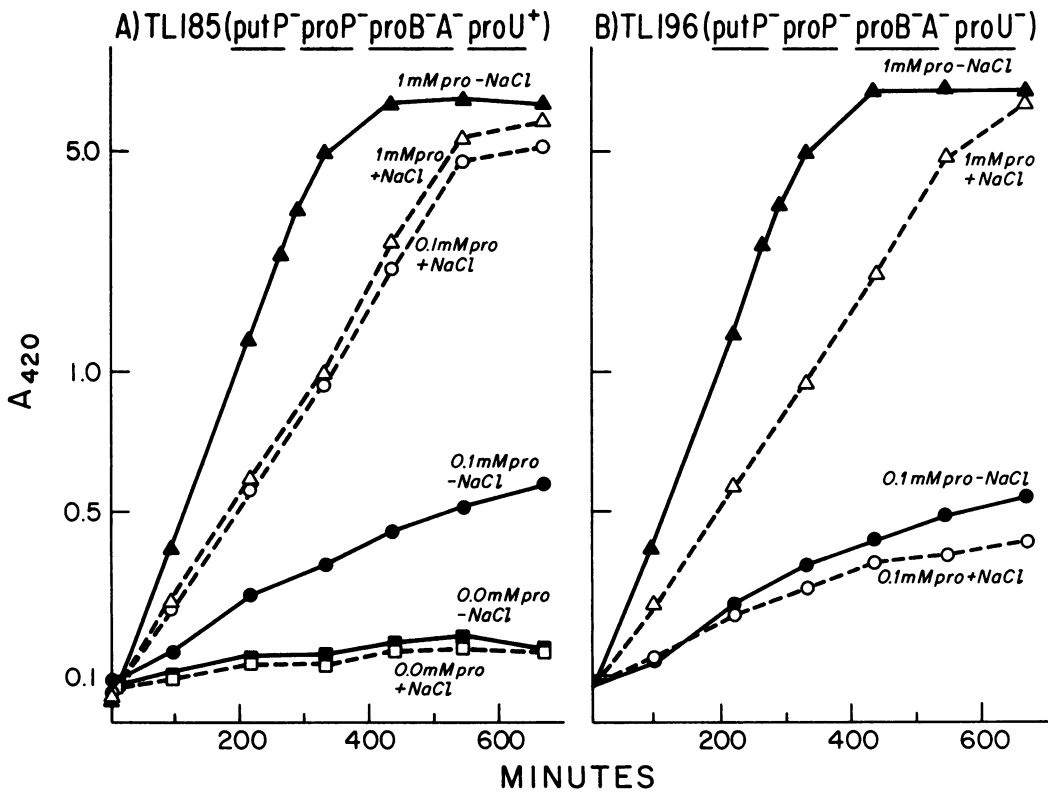

FIG. 3. The effect of *proU* mutation on the growth of *putP proP proBA* strains with various external concentrations of proline in the presence of 0.3 M NaCl. The optical density at 420 nm of cultures is plotted as a function of incubation time. Closed symbols and solid lines indicate growth in minimal medium; open symbols and broken lines indicate growth in minimal medium plus 0.3 M NaCl. Symbols: ■, □, growth without proline; ●, ○, growth with 0.1 mM proline; ▲, △, growth with 1 mM proline. (A) Strain TL185 (*putP proP proBA proU*$^+$); (B) strain TL196 (*putP proP proBA proU*).

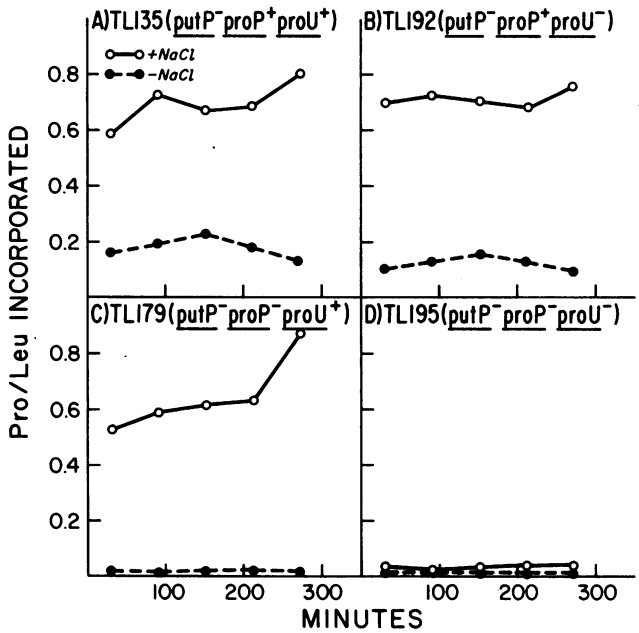

FIG. 4. The effect of osmotic stress on the incorporation of exogenous proline into protein of growing cells of proline transport mutants. Cells were grown in the presence of 0.05 mM [^{3}H]proline and 0.5 mM [^{14}C]leucine. Ratio of exogenous proline molecules to leucine molecules incorporated into protein is plotted as a function of incubation time. For experimental details, see the text. Symbols: ●, growth in minimal medium; ○, growth in minimal medium plus 0.3 M NaCl. (A) strain TL135 (*putP proP*$^+$ *proU*$^+$); (B) strain TL192 (*putP proP*$^+$*proU*); (C) strain TL179 (*putP proP proU*$^+$); (D) strain TL195 (*putP proP proU*). Ratios of the raw [^{3}H]proline/[^{14}C]leucine counts per minute above background in 0.1-ml samples at 30 min in minimal medium were: strain TL135, 384/48; strain TL192, 317/58; strain TL179, 55/53; strain TL195, 40/60; in minimal medium plus 0.3 M NaCl at 30 min they were: strain TL135, 996/41; strain TL192, 706/26; strain TL179, 480/22; strain TL195, 43/23. The counts incorporated increased approximately exponentially with time (data not shown), such that at 271 min in minimal medium, the ratios were: strain TL135, 2.15 × 10^4/3.44 × 10^3; strain TL192, 1.54 × 10^4/3.24 × 10^3; strain TL179, 3.78 × 10^3/3.26 × 10^3; strain TL195, 1.42 × 10^3/3.19 × 10^3; in minimal medium plus 0.3 M NaCl they were: strain TL135, 2.56 × 10^4/763; strain TL192, 2.47 × 10^4/847; strain TL179, 2.05 × 10^4/548; strain TL195, 1.11 × 10^3/612.

proU::Tn*10* and *srlA* or *tct*. However, *proU* is linked to *nalB*, which is also between *srl* and *tct* (19). Strain KS296 (*nalB*) was transduced to tetracycline resistance by a P22 lysate obtained from strain TL187 (*proU1655*::Tn*10*), and 66 of 100 transductants inherited the *nalB*$^+$ gene. These results show that *proU1655* is at approximately 59 map units of the chromosomal map, but the relative order of *nalB* and *proU* with respect to the outside markers *srl* and *tct* is not known.

DISCUSSION

I have presented data which indicate that the entry of proline into cells of *putP proP S. typhimurium* mutants (which are deficient in the two previously known proline permeases) is stimulated as a consequence of growth in media of elevated osmotic strength. Also, I identified a gene, *proU*$^+$, which is required for the stimulation of proline entry into such strains. One explanation for these observations could be that a third proline permease is expressed in response to osmotic stress, and the *proU*$^+$ gene is necessary for its expression. Alternatively, it is possible that exposure to media of elevated osmolarity increases the permeability of the cytoplasmic membrane to allow passive diffusion of proline and that the *proU*$^+$ gene product is necessary to confer increased permeability of the cell membrane. Although I have not rigorously ruled out the latter alternative, for a number of reasons I do not consider it to be very likely. First, the entry of proline into *putP proP* strains in the presence of 0.3 M NaCl, as measured by the dependence of the growth rate of a *proBA* derivative on the exogenous proline concentration, shows saturation kinetics such that relatively low concentrations (~0.02 mM) of proline are sufficient to sustain half maximal growth rate (Fig. 1). Second, although there is a stimulation of the incorporation of external proline into protein of a *putP proP proU*$^+$ strain which is abolished by the *proU* mutation, the

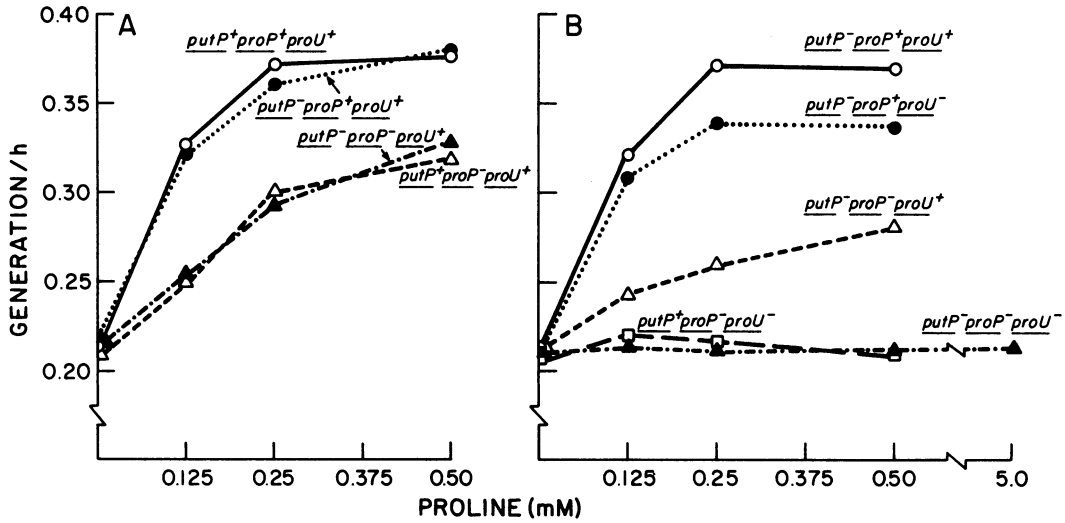

FIG. 5. The effect of proline permease mutations on the stimulation of growth rate by proline in media of inhibitory osmolarity. The growth rate of strains in minimal medium containing 0.65 M NaCl is plotted as a function of exogenous proline concentration. (A) Effect of *putP* and *proP* mutations. Symbols: O–O, strain TL1 (*putP*$^+$ *proP*$^+$ *proU*$^+$); ● · · · · ●, strain TL135 (*putP proP*$^+$ *proU*$^+$); △– – –△, strain TL143 (*putP*$^+$ *proP proU*$^+$); ▲– · –▲, strain TL145 (*putP proP proU*$^+$). (B) Effect of *proP* and *proU* mutations. Symbols: O–O, strain TL135 (*putP proP*$^+$ *proU*$^+$); ● · · · · ●, strain TL192 (*putP proP*$^+$ *proU*); △– – –△, strain TL179 (*putP proP proU*$^+$); ▲– · –▲, strain TL195 (*putP proP proU*); □– –□, strain TL199 (*putP*$^+$ *proP proU*).

incorporation of leucine is not affected either by osmotic stress or by the *proU* genotype (Fig. 4C and D). Third, the *proU* mutation has no recognizable phenotype in strains otherwise proficient in proline transport (i.e., *putP*$^+$ or *proP*$^+$), either in minimal medium or in media of elevated osmolarity, as measured by the sensitivity of strains to proline analogs (see Table 4), by the incorporation of exogenous proline or leucine into protein (Fig. 4A and B), and by the dependence of the growth rates of proline auxotrophic derivatives on the external proline concentration (data not shown).

I propose that a third proline permease is switched on in media of elevated osmotic strength. The *proU*$^+$ gene could be either a structural gene for a component of this permease or a regulatory gene required for its expression. In a preliminary communication, I referred to the same gene as *proT*$^+$ (7), but since that designation has been used to refer to a different gene involved in proline transport in *E. coli* (13), I now change the symbol to *proU*$^+$.

I constructed strains carrying all eight combinations of wild-type and mutant alleles of *putP*, *proP*, and *proU* and tested their sensitivities to Azt and Dhp (experimental details not shown). The results are summarized in Table 4. There are two main points to be made about these phenotypes. First, in minimal medium, *putP proP*$^+$ strains (irrespective of the *proU* genetype) are Azt^r Dhp^s, but *putP proP* (*proU*$^+$ or *proU*$^+$) strains are Azt^r Dhp^r. Second, in the presence of 0.3 M NaCl, *putP proP proU*$^+$ strains are Azt^s Dhp^s, but *putP proP proU* strains are Azt^r and Dhp^r (both in minimal medium and in the presence of 0.3 M NaCl). On the basis of these phenotypic differences, it can be concluded that the *proP* permease (PP-II) is distinct from the *proU* permease (PP-III). This conclusion is further supported by the mapping results that *proU* is at 59 map units of the *S. typhimurium* chromosomal map, whereas *proP* is at 92 map units (12).

There is an apparent discrepancy between my results (Table 4) and those obtained by Wood in

TABLE 4. Sensitivities of proline transport mutants to proline analogs[a]

Genotype			Minimal medium		Minimal medium plus 0.3 M NaCl	
putP	*proP*	*proU*	Azt	Dhp	Azt	Dhp
+	+	+	S	S	S	S
–	+	+	R	S	S	S
–	–	+	R	R	S	S
–	–	–	R	R	R	R
–	+	–	R	S	S	S
+	+	–	S	S	S	S
+	–	+	S	S	S	S
+	–	–	S	S	S	S

[a] S denotes sensitivity, and R denotes resistance to the indicated compounds

E. coli (22). She reported that *putP proP*$^+$ strains were Azt^r and Dhp^r (22), whereas I found that they were Azt^r but Dhp^s. The reason for this discrepancy is probably the fact that Wood used *putP proP*$^+$ strains which were proficient in proline dehydrogenase (*putA*$^+$), but the strains I used were *putA*. Since Dhp can be detoxified by proline dehydrogenase, *putPA proP*$^+$ strains are more sensitive to the analog than are *putPA*$^+$ *proP*$^+$ strains (22).

According to my results, the alleviation of osmotic inhibition by proline, which was discovered by Christian (3, 4), is mediated by the *proP*$^+$ and *proU*$^+$ genes. In *proP*$^+$ *proU* strains, the ability of proline to stimulate growth is slightly decreased; in *proP proU*$^+$ strains, the decrease is even more pronounced; and in *proP proU* strains, proline is not stimulatory at all. Britten and McClure found that in *E. coli*, the intracellular proline levels were elevated in direct proportion to increases in the osmolarity of the growth medium when proline was present externally (2). Subsequently, Kaback and Deuel reported that proline transport into cell-free membrane vesicles of *E. coli* was enhanced upon exposure to media of elevated osmolarity (10). This stimulation of proline transport could be due to the stimulation of PP-III. At present, I have no experimental data which indicate whether the stimulation of PP-III occurs at the level of functioning of a preexisting proline transport system or at the level of increased synthesis of component(s) of the transport system or both. There is one example of a transport system whose expression is regulated by osmotic stress: the expression of the *kdp* operon, which codes for a potassium transport system in *E. coli*, is increased in response to elevation of the osmotic stress of the medium (11). Since both PP-II and PP-III mediate the stimulatory effects of proline in media of inhibitory osmolarity, it is possible that PP-II is also stimulated in response to osmotic stress.

It is not clear why high intracellular proline levels result in growth rate stimulation under conditions of osmotic inhibition. One reason might be that proline is a general osmotic balancer whose internal levels could be regulated in response to osmotic stress by the activity of PP-II or PP-III when it is present in the medium. Therefore, it may serve to maintain the osmotic strength of the cell interior to equal or exceed that of the exterior and thus prevent osmotic dehydration of the cytoplasm. An alternative hypothesis was formulated by Schobert (17) and Schobert and Tschesche (18), according to whom proline is not a general osmotic balancer but rather has special interactions with membranes or proteins to stabilize their conformations in solutions of low water activity. Presently, there is not sufficient evidence to determine which of the two explanations is correct. But Schobert's hypothesis is intriguing because it implies that high intracellular proline levels would in general be sufficient to confer increased osmotolerance.

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