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proP-Mediated Proline Transport Also Plays a Role in *Escherichia coli* Osmoregulation

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The growth of *Escherichia coli* strains in media having elevated osmolarity was promoted in the presence of low concentrations of the L-proline analog 5-hydroxy-L-pipecolic acid. The osmoprotective ability of this compound was correlated with the presence in these strains of a functional *proP*$^+$ gene. The results suggest that the *proP*-mediated transport of L-proline (in addition to that by *proU*) is important in osmoregulation. *proP*::*lac* operon fusions were used to demonstrate that this gene shows limited induction of expression upon growth in media having elevated osmolarity and that it is transcribed clockwise on the chromosome.

It has been known for some time that submillimolar concentrations of L-proline promote the growth of several species of *Enterobacteriaceae* in media having otherwise inhibitory osmolarity (4–7, 9, 15). The osmoprotective effect of L-proline in these strains is believed to be related to the enhanced transport of L-proline into cells subjected to water stress (6, 7, 9, 12) and to its role as an intracellular "compatible solute" in maintaining the cell turgor pressure under these conditions (15, 16). Csonka and co-workers identified a gene called *proU* in *Salmonella typhimurium* which codes for an L-proline transport system active in cells grown in media having elevated osmolarity (6, 7). I recently showed that a homologous gene also exists in *Escherichia coli*, whose transcriptional expression is induced 400-fold upon growth in media having elevated osmolarity (9).

Both *E. coli* and *S. typhimurium* have two other transport systems for L-proline coded for by the *putP* and *proP* genes (17, 19, 20, 22). In the course of the earlier studies on *proU* with both of these bacteria (6, 9), mutations in *proP* were also found to contribute to the loss of the osmoprotective ability of L-proline; however, it could not be established whether this reflected a secondary effect caused by an increased load on the osmoregulatory *proU*-coded permease or whether *proP*-mediated transport of L-proline was in itself important in the adaptational response. The results of the studies reported here suggest that the latter case is indeed true.

The strains used in this study were derivatives of *E. coli* K-12 (Table 1). The bacteriophage Mu dl(*lac* Ap), obtained by temperature induction of MAL103, was described by Casadaban and Cohen (3). The media used included minimal A and LB media (18) and the low-osmolarity K medium of Kennedy (13). Tetracycline, ampicillin, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 3,4-dehydro-DL-proline (DHP), and 5-hydroxy-L-pipecolic acid (5-HPi) (Sigma Chemical Co., St. Louis, Mo.) were used at final concentrations of 15 μg/ml, 25 μg/ml, 25 μg/ml, 0.4 mM, and 0.5 mM, respectively. The methods used for P1 *kc* transduction and conjugation were described earlier (9, 18). β-Galactosidase assays were done by the method of Miller (18), and enzyme specific activity was expressed in units as defined therein.

Osmoprotective effect of 5-HPi is manifest only in *proP*$^+$ strains. In the course of studies aimed to test for possible differences in the substrate specificities of the PutP, ProP, and ProU transport systems, the six-membered-ring analog of L-proline, 5-HPi, was found to exhibit interesting properties. Preliminary results indicated that this compound (at 1 mM concentration) was not an inhibitor of *E. coli* growth, and that, like L-proline, it could promote their growth in media having elevated osmolarity. The osmoprotective abilities of L-proline and 5-HPi were compared by the testing for growth on plates of media having elevated osmolarity of a panel of eight isogenic strains with different combinations of *putP*, *proP*, and *proU* alleles (Table 2). As has been reported earlier (9), the osmoprotective effect of L-proline was maximal in *proP*$^+$ *proU*$^+$ strains, somewhat less prominent in *proP proU*$^+$ or *proP*$^+$ *proU* strains, and absent in the *proP proU* strains. In contrast, 5-HPi promoted the growth of only the *proP*$^+$ strains, irrespective of their *putP* or *proU* genotype. Notably, the *proP proU*$^+$ strains (GJ134 and GJ167) were not osmoprotected by 5-HPi, although they did show osmoprotection by L-proline. The results of the plate tests were also confirmed by measurements of generation time in batch culture; in particular, the growth rates (h^{-1}) of strain GJ130 (*proP*$^+$ *proU*$^+$) in the medium having elevated osmolarity (minimal A plus 0.65 M NaCl) supplemented with L-proline or 5-HPi were 0.26 and 0.14, respectively, whereas those of GJ134 (*proP proU*$^+$) in the same medium were 0.21 and <0.02, respectively. (The growth rates of both strains in this medium unsupplemented with L-proline or 5-HPi were also <0.02 h^{-1}.) Osmoprotection by 5-HPi was also observed in another *E. coli* K-12 strain tested (W3110) and in *S. typhimurium* LT2 (data not shown).

The observation that 5-HPi can function as an osmoprotective agent in *E. coli*, but only in strains that are *proP*$^+$, suggested that this analog of L-proline is a substrate for the ProP transport system but not for ProU, and that, by analogy, ProP-mediated transport of L-proline is in itself probably important in the exhibition of the osmoprotective effect of this imino acid in *E. coli* (in addition to the well-established role for *proU* in this function [9]). Although studies on the metabolism of 5-HPi in procaryotes have not been reported, it is likely that it is metabolically inert, as indeed has been shown in studies with this compound on higher-plant and animal cell systems (11, 21). In light of this fact, the present results suggest that the osmoprotective function of L-proline (or at least that part of it which is dependent on ProP-mediated uptake) is also not related to its metabolic functions in the cell, namely, to serve as a source for C or N in growth or as a substrate in protein synthesis.

Regulation of *proP* expression. Earlier, it was suggested

TABLE 1. *E. coli* K-12 strains used

Strain	Genotype[a]	Source or derivation
W3110	F⁻	J. Pittard
MC4100	F⁻ Δ(*argF-lac*)*U169 rpsL150 relA1 araD139 flbB5301 deoC1 ptsF25*	2
MAL103	F⁻ *araB*::Mu cts *araD139* Δ(*gpt-lac*)*5 rpsL* [Mu dl(*lac* Ap)]	3
CSH57	F⁻ *purE trp his argG met ilv leu thi ara lacY gal malA xyl mtl rpsL*	18
JP3302	F⁺ *purE trp his argG met ilv leu thi xyl mtl pheR372* Δ(*argF-lac*)*U169 zjd-351*::Tn*10 rpsL* (λ p*pheA-lac*)	10
GJ11	MC4100 *proU224*::*lac* [λ p1 (209)]	9
GJ130	MC4100 Δ*putPA101* Δ(*pyr-76*::Tn*10*)	From GJ125 (9), by Tet^s selection
GJ134	MC4100 Δ*putPA101 proP222* Δ(*pyr-76*::Tn*10*)	9
GJ156	GJ11 Δ*putPA101* Δ(*pyr-76*::Tn*10*)	From GJ121 (9), by Tet^s selection
GJ157	GJ11 Δ*putPA101 proP221* Δ(*pyr-76*::Tn*10*)	9
GJ167	MC4100 *proP222*	From GJ134, by P1 *kc* transduction to Ura$^+$ Put$^+$
GJ168	GJ11 *proP221*	From GJ157, by P1 *kc* transduction to Ura$^+$ Put$^+$
GJ182	GJ130 *proP226*::Mu dl(*lac* Ap)	This study
GJ183	GJ130 *proP227*::Mu dl(*lac* Ap)	This study
GJ184	GJ130 *proP228*::Mu dl(*lac* Ap)	This study
GJ185	GJ130 *proP229*::Mu dl(*lac* Ap)	This study
GJ186	GJ130 *proP230*::Mu dl(*lac* Ap)	This study
GJ188	GJ130 *proP232*::Mu dl(*lac* Ap)	This study

[a] Genotype designations used in this study are those described by Bachmann (1). Allele numbers are indicated where they are known. The *proU224* allele was described as *osrA2* in an earlier study (9).

that a gene important in osmoregulation must show osmoresponsivity in its expression or that its product must show osmoresponsivity in its biological activity (9). Strains having *proP-lac* operon fusions were constructed in this study to examine whether *proP* expression in *E. coli* is influenced by the osmolarity of the growth medium.

proP::*lac* operon fusions were obtained by infection of a Δ*lac putPA proP*$^+$ strain, GJ130, with a Mu dl(*lac* Ap) phage lysate, and selection was made for Amp^r DHP^r colonies; it is known that selection for DHP^r in a *putPA* strain permits the isolation of *proP* mutants (9, 20). The strains so obtained were tested for their Lac phenotype on X-gal indicator plates, and Lac$^+$ and Lac$^-$ colonies (obtained in approximately a 1:1 ratio) were presumed to represent the two orientations in which Mu dl(*lac* Ap) could insert into *proP*. The following observations lent additional support to this interpretation. Three Lac$^+$ strains (GJ182, GJ183, and GJ186) and one Lac$^-$ strain (GJ188) were transduced to Tet^r with a P1 *kc* lysate prepared on JP3302 (*proP*$^+$ *zjd-351*::Tn*10*); between 40 and 50% of the Tet^r transductants were shown, in each instance, to have concomitantly become Amp^s DHP^s (data not shown). The *zjd-351*::Tn*10* insertion was earlier shown to be 40% cotransducible with the *proP* gene (9). This result, therefore, indicated that all of these strains are indeed *proP*::Mu dl(*lac* Ap) monolysogens. The F-prime plasmid F(Ts)*114 lac* was then introduced into the *zjd-351*::Tn*10* derivatives of GJ182, GJ186, and GJ188 and used (with strain CSH57 serving as the recipient in chromosome mobilization experiments to determine the orientation of the Mu dl(*lac* Ap) insertion in these strains. The F-prime plasmid mobilized the chromosome of the first two strains (both Lac$^+$) in a clockwise direction, with a 400:1 ratio of recovery of Ade$^+$ (*purE*$^+$) to Ilv$^+$ exconjugants. Furthermore, approximately 60% of the Ade$^+$ recombinants in each of these two crosses were also found to have inherited the *zjd-351*::Tn*10* allele of the donor strain, indicating that this Tn*10* insertion was also being mobilized as an early marker in these two strains. In contrast, in the experiment in which the third *proP*::*lac* strain (GJ188 Lac$^-$) was used as donor, Ilv$^+$ recombinants were obtained at a 1,000-fold-higher frequency than were Ade$^+$ recombinants, suggestive of a counterclockwise direction of mobilization in this strain. These results not only served to confirm the notion that the Mu dl(*lac* Ap) insertions in *proP* are in opposing orientations in the Lac$^+$ and Lac$^-$ lysogens, respectively, but also indicated that *proP* is transcribed clockwise on the *E. coli* chromosome and that *proP* lies immediately counterclockwise of *zjd-351*::Tn*10* (and of *pheR* [10]) on the linkage map. All of the *proP*::*lac* strains tested also showed osmoprotection with L-proline but not with 5-HPi, in accord with the results described above.

The specific activity of β-galactosidase in several Lac$^+$ *proP*::*lac* fusion strains was measured after growth in K medium (70 mosM) with or without 0.4 M NaCl (Table 3). For each of the strains, enzyme specific activity in cultures grown in K medium plus 0.4 M NaCl was 70 to 90% higher than that of cultures grown in the low-osmolarity medium, and this difference was consistent and reproducible. β-Galactosidase specific activity in these strains was not affected by the addition of 5 mM L-proline to the growth

TABLE 2. Osmoprotective effect of L-proline and 5-HPi on *E. coli* strains

Strain	Genotype	Growth on minimal A medium + 0.65 M NaCl supplemented with:[a]		
		None	L-Proline (0.5 mM)	5-HPi (0.5 mM)
MC4100	*putP*$^+$ *proP*$^+$ *proU*$^+$	−	+ + +	+ +
GJ11	*putP*$^+$ *proP*$^+$ *proU*	−	+ + +	+ +
GJ130	*putP proP*$^+$ *proU*$^+$	−	+ + +	+ +
GJ134	*putP proP proU*$^+$	−	+ + +	−
GJ156	*putP proP*$^+$ *proU*	−	+ +	+ +
GJ157	*putP proP proU*	−	−	−
GJ167	*putP*$^+$ *proP proU*$^+$	−	+ +	−
GJ168	*putP*$^+$ *proP proU*	−	−	−

[a] Growth was scored on a scale of 4 (from −, no growth, to + + +, good growth) after a radial streaking of strains on agar medium and incubation at 37°C for 60 h.

TABLE 3. Effect of osmolarity on β-galactosidase specific activity in *proP*::*lac* strains

Strain	β-Galactosidase sp act (U) after growth in:[a]	
	K medium	K medium + 0.4 M NaCl
GJ182	138	216
GJ183	135	221
GJ184	130	192
GJ185	108	197
GJ186	37	82

[a] Strains were grown for at least 10 generations in the medium specified before enzyme specific activity measurements were made.

medium (data not shown). The specific activity of β-galactosidase in two other operon fusion strains tested, in which the *lac* genes had been fused to *aroP* and *tyrP* (genes coding for two other aminoacyl permeases in *E. coli*), was also not affected by changes in the osmolarity of the growth medium (data not shown), indicating that the effect on *proP* is probably not a nonspecific one.

These results suggest that the expression of *proP* is induced to a limited extent by an increase in the osmolarity of the growth medium. The magnitude of induction is, however, much less than that of other osmoregulatory genes such as *proU* or *kdp* (9, 14). Nonetheless, in the light of its role in contributing to the L-proline osmoprotective effect in *E. coli*, the osmoresponsive induction of *proP* expression may be an important component of the overall adaptational response. The *proP* gene in *S. typhimurium* was also shown recently to be induced to a similar small extent by growth in media having elevated osmolarity (7). These workers also showed that the transport activity of the ProP gene product(s) is enhanced, at a posttranscriptional level, by changes in cell turgor pressure similar to that which has been described for the Kdp-mediated K^+ transport system in *E. coli* (8).

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