

Genetics of L-Proline Utilization in *Escherichia coli*

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L-Azetidine-2-carboxylate (AC) and 3,4-dehydro-D,L-proline (DHP) are toxic L-proline analogs that can be used to select bacterial mutants defective for L-proline transport. Mutants resistant to AC and DHP are defective for proline transport alone (*putP* mutants), and mutants resistant to AC but not to DHP are defective both in *putP* and in the closely linked proline dehydrogenase gene *putA*. Proline dehydrogenase oxidizes DHP but not AC, probably detoxifying the former compound. These observations were exploited in preparing an otherwise isogenic set of *Escherichia coli* K-12 strains with well-defined defects in the *putP* and *putA* genes. The results of this study suggest that the genetic and biochemical characteristics of proline utilization in *E. coli* K-12 are closely analogous to those of *Salmonella typhimurium*.

Proline can serve as sole source of carbon or nitrogen for the growth of *Escherichia coli* K-12. It is converted via two oxidation reactions to L-glutamate. The oxidation of L-proline to Δ^1 -pyrroline carboxylate is linked with the reduction of molecular oxygen, and the further oxidation of Δ^1 -pyrroline carboxylate to L-glutamate is NAD⁺ linked. Production of proline dehydrogenase and of the active transport system proline porter I (PP-I) is controlled by catabolite repression and induction by proline (11, 19). A second proline porter, PP-II, is probably also present (8). Although a number of *E. coli* mutants defective for proline utilization have been isolated, no systematic analysis of the proline utilization (*put*) genes has been presented.

The genetic characteristics of L-proline utilization in *Salmonella typhimurium* have been analyzed thoroughly by Roth and his colleagues (8, 11, 12; R. Menzel, Ph.D. thesis, University of California, Berkeley, 1980). Proline dehydrogenase is encoded in *putA*, and mutations at the locus *putP* inactivate a proline transport system analogous to PP-I of *E. coli* (12). The *putP* and *putA* genes are adjacent at 22 min on the chromosome map, and the *putA* gene product also serves as the repressor controlling the expression of both *putP* and *putA* (Menzel, Ph.D. thesis). Although their regulation is coordinate, the *put* genes seem to be transcribed from separate promoters (Menzel, Ph.D. thesis).

A second L-proline transport system, PP-II, has been examined both genetically and biochemically in *S. typhimurium* (1, 8). PP-II is inactivated by mutations at *proP*, which is located at 92 min on the chromosome map. Although PP-I and PP-II share almost identical

substrate specificity, PP-II shows a 100-fold higher K_m for proline than does PP-I, and their regulation also differs. PP-II is induced when amino acid-auxotrophic bacteria are starved for their auxotrophic requirement, probably as part of the stringent response (8). *proP* defects in *S. typhimurium* can be complemented by an *E. coli* episome including the corresponding chromosome segment, implying that PP-II is also present in *E. coli* (Menzel, Ph.D. thesis).

Mutants defective for proline transport have most frequently been isolated by selecting bacteria resistant to growth inhibition by either of the toxic L-proline analogs L-azetidine-2-carboxylate (AC) or 3,4-dehydro-D,L-proline (DHP). All of the well-characterized mutants selected in this way have been defective at *putP*, thereby lacking PP-I activity (12, 19; Menzel, Ph.D. thesis). Tristram and Neale found that L-proline transport mutants of *E. coli* selected as AC resistant were not cross-resistant to DHP, but those selected as resistant to DHP were cross-resistant to AC (17). They thus found proline transport mutants with two distinct analog resistance phenotypes. The chromosomal locations of these mutations were not determined. This paper describes the further genetic analysis of L-proline utilization in *E. coli* and explains the biochemical and genetic bases for the two analog resistance phenotypes observed by Tristram and Neale.

MATERIALS AND METHODS

Materials. All reagents used were from the sources stated previously, and culture media were prepared as described before (20).

Strains. The bacterial strains prepared and used

during this study, all derivatives of *E. coli* K-12, are listed in Table 1. Strains CSH4 and CSH59 are part of a collection from Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. Strain RM2 was a gift from R. Menzel (University of Utah, Salt Lake City). Bacteriophage λ c1857 *rex::Tn5* Oam29 Pam80 b221, used for Tn5 mutagenesis, was a gift from J. D. Friesen (York University, Toronto, Ontario, Canada), and bacteriophage P1 CM *cb*-100 was a gift from J. J. Anderson (University of Michigan, Ann Arbor). The derivations of all other strains are described in Results.

Tn5 mutagenesis. Stocks of mixed Tn5 insertion mutants of strain CSH4 were prepared essentially as described by Shaw and Berg (14). LB medium (9) replaced tryptone, and phenotypic expression was for 2 h at 30°C. Mutants were selected by spreading approximately 10^{10} bacteria per plate on LB medium containing kanamycin sulfate (25 μ g/ml) and sodium pyrophosphate (2.5 mM). After overnight incubation at 37°C, the lawns of insertion mutants were suspended in 1 ml of LB medium. A 1-ml portion of 50% glycerol solution was added, and the mutant stocks were stored at -70°C for subsequent mutant selection.

Mutant selection. (i) **Proline dehydrogenase.** Approximately 5,000 Tn5 insertion mutants of strain CSH4 were spread on a single triphenyltetrazolium chloride (TTC)-proline indicator plate. The white colonies were identified, purified, and tested as described below to resolve proline dehydrogenase mutants from proline transport mutants and auxotrophs.

(ii) **Proline analog-resistant mutants.** In selection 1, 10^5 Tn5 insertion mutants suspended in 0.85% NaCl (wt/vol) solution were spread on minimal salts medium containing D-glucose and NH_4Cl as carbon and nitrogen sources and AC at 25 μ g/ml or DHP at 10 μ g/ml. After 48 h of incubation at 37°C, resistant colonies were picked, purified by restreaking on selective medium, and tested as described below.

In selection 2, a fresh overnight culture of strain CSH4 in LB medium was harvested by centrifugation and suspended in 0.85% NaCl (wt/vol). A total of 5×10^6 (AC resistance) or 2×10^6 (DHP resistance) bacteria were spread on the selective media described above (selection 1). After 48 h of incubation at 37°C, the AC-resistant colonies (approximately 300 per plate) were replicated onto TTC-proline indicator medium. Those white colonies appearing after a further 24 h of incubation at 37°C were picked, purified by restreaking on the indicator medium, and tested as described below. DHP-resistant mutants were purified and tested without screening.

Phenotypic tests. The mutant phenotypes described in Tables 2 and 3 were determined by the following tests, each of which used a fresh overnight culture of the mutant strain in LB medium (9) as the inoculum.

(i) **TTC-proline indicator medium.** The capacity to grow on proline as carbon or nitrogen source was tested by using TTC-containing indicator medium (4) with L-proline (2 mg/ml), tryptophan (50 μ g/ml), and thiamine (1 μ g/ml) added (TTC-proline medium). Strains able to utilize L-proline form red colonies on this medium, and those unable to utilize L-proline are white. Strains that formed white colonies because they had acquired an additional auxotrophic requirement

TABLE 1. *E. coli* K-12 strains^a

Strain	Genotype or phenotype
CSH4	F ⁻ <i>trp lacZ rpsL thi</i>
CSH59	F ⁻ <i>trp pyrC rpsL thi</i>
JT18	CSH4 <i>putA1::Tn5</i>
JT27	CSH4 <i>Km'</i> (Tn5)
JT28	CSH4 <i>putP3::Tn5</i>
JT31	CSH4 <i>putA1::Tn5</i>
JT34	CSH4 <i>putP3::Tn5</i>
RM2	CSH4 Δ (<i>putPA</i>)100
WG42	CSH4 <i>putP4</i>
WG69	CSH4 <i>putP5</i>
WG78	CSH4 AC ^r

^a The genetic nomenclature is that of Bachmann and Low (2).

were identified by streaking on minimal medium and discarded.

(ii) **Proline analog sensitivity.** Sensitivity to the toxic L-proline analogs AC and DHP was tested by the radial streak method described before (20).

(iii) **Proline dehydrogenase.** Strains were screened for proline dehydrogenase activity essentially as described by Dendinger and Brill (6). The strains to be tested were grown on D-glucose and NH_4Cl as carbon and nitrogen sources, with glycyl-L-proline as an inducer.

Phage lysates and transductions. λ and P1 lysogens and λ and P1 lysates were prepared essentially as described by Miller (9). Bacteriophage P1-mediated transductions were also performed as described previously (9). Kanamycin-resistant transductants were selected at 37°C on LB medium containing kanamycin sulfate (25 μ g/ml) and sodium pyrophosphate (2.5 mM) after 30 min of phenotypic expression in LB medium at 25°C. Uracil prototrophic transductants were selected at 37°C on minimal salts medium containing D-glucose and NH_4Cl as carbon and nitrogen sources. All transductants were purified by restreaking on the selective medium before growth in LB medium and testing as described in Results.

Transport assays. Bacterial cultures were prepared and initial rates of amino acid uptake were measured by the filtration assay described before (20).

Quantitative dehydrogenase assays. (i) **Whole cells.** The proline dehydrogenase activity of whole cells was determined by a modification of the assay, employing o-aminobenzaldehyde (oAB), of Dendinger and Brill (6). It was performed as previously described (19).

(ii) **Membrane vesicles.** The dehydrogenase activity of membrane vesicles was determined either via the reaction of Δ^1 -pyrroline carboxylate with oAB (oAB assay [16]) or via the reduction of dichlorophenolindophenol (DCPIP assay [13]). In each case, a 2.5-ml assay mixture was prepared containing sodium cacodylate (0.2 M, pH 6.8), substrate (60 mM), and membranes (0.2 to 0.4 mg of membrane protein). For the oAB assay, the assay mixture also contained oAB (4 mM), and for the DCPIP assay it contained potassium cyanide (1 mM), phenazine methosulfate (0.2 mM), and DCPIP (0.05 mM). In each case the membranes were preincubated in the reaction mixture for

TABLE 2. Predicted phenotypes and genotypes of proline utilization mutants

Test ^a	Predicted phenotype ^b			
	Parent	I	II	III
TTC-proline	+	—	—	—
Analog resistance				
AC	S	R	R	SS
DHP	S	R	S	SS
Proline dehydrogenase	+	+	—	—
Predicted genotype	<i>putP⁺ putA⁺</i>	<i>putP putA⁺</i>	<i>putP putA</i>	<i>putP⁺ putA::Tn5</i>

^a The phenotypic tests and their implications are described in the text.^b The phenotypes are sensitive (S), resistant (R), and supersensitive (SS).TABLE 3. Proline utilization mutants of strain CSH4^a

Mutagen	Selection/ screening	No. of mutants per phenotype		
		I	II	III
None	AC	35	30	0
None	DHP	96	0	0
Tn5	AC	98	0	0
Tn5	DHP	100	0	0
Tn5	TTC	1	0	3

^a Mutants were selected and their phenotypes were determined as described in the text.

3 min before the addition of substrate. The increase in absorbance at 443 nm (oAB assay) or the decrease in absorbance at 600 nm (DCPIP assay) was recorded at 25°C, using a Beckman Acta CIII spectrophotometer. Initial reaction rates were calculated assuming extinction coefficients of $2.71 \times 10^3 \text{ cm}^{-1} \text{ M}^{-1}$ for the oAB assay (16) and $1.61 \times 10^4 \text{ cm}^{-1} \text{ M}^{-1}$ for the DCPIP assay (5). The reaction rates determined with DCPIP were corrected by subtracting the rate of dye reduction by substrate in the absence of enzyme, a correction that was always less than 10% of the enzyme-catalyzed reaction rate.

Membrane preparations. Washed membranes were prepared from bacteria grown on succinate, NH_4Cl , and L-proline as carbon and nitrogen sources, using the French pressure cell as described by Menzel (Ph.D thesis). The membrane pellet was suspended in 20 ml of sodium cacodylate (0.5 M, pH 6.8) per g (wet weight) of pellet, and samples were frozen at -70°C . Freezing and thawing the membrane preparation once did not alter enzymatic activity, and no loss of activity was observed during 3 weeks of storage.

Protein assays. The protein content of cell and membrane suspensions was determined by the method of Lowry et al. (7), using bovine serum albumin as a standard.

RESULTS

During initial experiments designed to select proline transport-defective mutants of *E. coli*, we also observed the two distinct analog resistance phenotypes originally reported by Tristram

and Neale (17). Our data suggested that each phenotype was due to a single mutation and that both mutations were closely linked to *pyrC* (B. Sullivan and J. Wood, unpublished data). The selection of AC^r mutants from a Tn5-mutagenized stock of strain CSH4 yielded only the AC^r DHP^r phenotype.

Consideration of these observations led to the following hypothesis: AC is a substrate for PP-I and PP-II but not for proline dehydrogenase, so its toxicity is a direct function of L-proline uptake activity and is independent of proline dehydrogenase activity. DHP is a substrate for PP-I, PP-II, and proline dehydrogenase, and it is detoxified by oxidation. DHP toxicity is therefore a direct function of proline uptake activity and an inverse function of proline dehydrogenase activity. AC and DHP are known to be substrates for both PP-I and PP-II (1, 19), and AC is not a substrate for proline dehydrogenase (13; Menzel, Ph.D. thesis). If the stated hypothesis is correct, the AC^r DHP^r phenotype would result from a reduction in transport activity alone, whereas the AC^r DHP^s phenotype would result from reductions in both transport and dehydrogenase activity.

Selection of proline utilization mutants. Both spontaneous and Tn5 insertion mutants were isolated (see Materials and Methods). Each mutant was characterized by testing its proline analog sensitivity, its response on TTC-proline indicator medium, and its ability to oxidize proline to Δ^1 -pyrroline carboxylate, placing it within one of the three phenotypic classes defined in Table 2. The quantitative results of the mutant selections are described below and summarized in Table 3.

If *E. coli* possesses *putPA* and *proP* loci exactly analogous to those of *S. typhimurium*, analog-resistant mutants selected from a strain that is wild type with respect to proline utilization will most likely be defective at *putP*. Transport system PP-I shows constitutive activity under the conditions of the selection that is sever-

alfold greater than the PP-II activity observed under the same conditions (1, 19). Nonetheless, PP-II is expected to provide a significant route of entry for DHP and AC in *putP* strains. Bacteria also lacking the detoxifying activity of proline dehydrogenase (*putP putA*) will therefore retain sensitivity to growth inhibition by DHP.

Spontaneous mutants. Spontaneous AC^r mutants of strain CSH4 would be expected to fall into two classes: mutants with reduced transport but normal proline dehydrogenase activity (*putP putA*⁺) would be resistant to AC and DHP (phenotype I of Table 2), whereas mutants with reduced transport and dehydrogenase activity (*putP putA*) would be resistant to AC but not to DHP (phenotype II of Table 2). Roughly equal numbers of mutants in each of these phenotypic classes were observed when AC^r mutants were selected (Table 3). Strain WG69 is a putative *putP putA*⁺ mutant and RM2 is a putative *putP putA* mutant selected from CSH4 as AC resistant by Rolf Menzel. These two strains were representatives of the two phenotypic classes that were retained for subsequent biochemical testing.

The selection for spontaneous AC-resistant mutants included a screening step to identify those mutants that gave white colonies on TTC-proline indicator medium (approximately 10% of those identified). This step was included since large numbers of spontaneous mutants arise that are AC resistant but retain normal proline utilization capacity. They apparently possess a mutation causing proline overproduction (17; Menzel, Ph.D. thesis). Strain WG78, an example of an AC-resistant strain with normal proline utilization capacity, was retained for biochemical analysis. The frequency of mutation to AC resistance with defective proline utilization was found to be approximately 2×10^{-6} .

Spontaneous, DHP-resistant mutants of strain CSH4 are expected to be of a single class: they should possess reduced proline transport but normal proline dehydrogenase activity (*putP putA*⁺) and should be cross-resistant to AC (phenotype I of Table 2). The 96 DHP-resistant mutants isolated did indeed fall into this category (Table 3), and WG42 is an example of such a strain. It was not necessary to use a screening step on TTC-proline indicator medium as part of the DHP selection since all of the mutants identified in this way were defective for L-proline utilization. The frequency of mutation to DHP resistance was found to be 2×10^{-7} .

Tn5 insertion mutants. If the *putP* and *putA* genes are transcribed from separate promoters, no polarity should be observed for spontaneous or Tn5 insertion mutants. All sponta-

neous *putP putA* mutants should be deletions, and no *putP putA* mutants should be obtained by Tn5 insertions within the *put* region. Transposon insertion mutants selected as either AC or DHP resistant should therefore retain proline dehydrogenase and be cross-resistant to the other analog (phenotype I of Table 2). Ten AC- and 10 DHP-resistant mutants were isolated from each of 10 Tn5 mutant stocks of strain CSH4. Only 8 of the 100 mutants selected as AC resistant were of the predicted phenotype. The other two AC resistant mutants retained DHP sensitivity, proline dehydrogenase activity, and proline utilization capacity (the phenotype of the putative proline-overproducing strains). These strains may be *argD::Tn5* mutants (3). Each of the 100 DHP-resistant mutants isolated by Tn5 mutagenesis was also AC resistant and defective for proline utilization, retaining proline dehydrogenase activity as predicted (Tables 2 and 3). The analog-resistant mutants arose from the Tn5-mutagenized stocks at frequencies between 2×10^{-4} and 6×10^{-4} under all of the selective conditions described. Strain JT28 was retained as an example of such a mutant.

It has been implied that the *putA* gene product of *S. typhimurium* is the repressor controlling *putP* and *putA* (Menzel, Ph.D. thesis), and we have suggested that, as proline dehydrogenase, it also detoxifies DHP. If the *putA* gene product of *E. coli* is also a repressor protein, then Tn5 insertion mutants lacking it should be supersensitive to both AC and DHP due to constitutive expression of PP-I and supersensitive to DHP due to reduced dehydrogenase activity (phenotype III of Table 2). (Note that point mutations in proline dehydrogenase eliminating its enzymatic but not its repressor activity would be expected to yield the phenotype AC^r DHPsm). Eleven Tn5 mutant stocks of strain CSH4 were screened for mutants yielding white colonies on TTC-proline indicator medium. Of 25 putative proline utilization mutants identified in this way, one was a proline transport mutant (phenotype I of Table 2) and three independent mutants were identified that lacked proline dehydrogenase and were supersensitive to both AC and DHP (phenotype III of Table 2). Strain JT18 was retained as an example of a proline dehydrogenase insertion mutant.

Chromosome map locations of the proline utilization defects. The mutant phenotypes observed as a consequence of the experiments described above were in full accord with predictions based on the reported genetics of proline utilization in *S. typhimurium* (11, 12; Menzel, Ph.D. thesis) and with the hypothesis that proline dehydrogenase detoxifies DHP. The *put* genes of *S. typhimurium* are adjacent and

are located between *pyrC* and *pyrD* at 21.5 min on the chromosome map. We have shown in an earlier study that a proline transport mutation in *E. coli* was 22% linked with *pyrC* by bacteriophage P1-mediated transduction (19). Strain RM2 is a *putPA* deletion mutant of strain CSH4 selected as AC resistant. The proline utilization defect of this strain is complemented by *S. typhimurium* episome F(Ts)'601 *put*⁺ *zcc-628*::Tn5 but not by analogous episomes bearing only intact *putP* (F(Ts)'601 *putA841*::Tn5) or *putA* (F(Ts)'601 *putP853*::Tn5) genes (Menzel, Ph.D. thesis and unpublished data). These observations reinforce the view that the *put* genes of *E. coli* and *S. typhimurium* are very similar in location and organization.

To directly test the map locations of the *putP*::Tn5 and *putA*::Tn5 mutations isolated during this study, we have determined their linkage to *pyrC*. Each mutation was first moved into an unmutagenized isolate of strain CSH4 by transduction. Strains JT18 (phenotype III of Table 3), JT28 (phenotype I of Table 3), and JT27 (a *put*⁺ Tn5 insertion strain) were used as donors, and the kanamycin-resistant transductants were characterized by the tests described in Table 2. Only 20 of 24 transductants from strain JT18 and 16 of 24 transductants from strain JT28 showed the proline utilization defects of the donor strains. None of the transductants from strain JT27 was defective for proline utilization. These data suggest that the donor strains contained multiple Tn5 insertions or Tn5 underwent transposition during these transductions (15) or both. Strains JT31 (from JT18) and JT34 (from JT28) were among the transductants isolated.

Strains JT31 and JT34 were used as donors in the P1-mediated transduction of strain CSH59 (*pyrC*) to uracil prototrophy. The transductants were analyzed for kanamycin sensitivity and for proline utilization phenotype, using the tests outlined in Table 2. Of 96 transductants from strain JT31, 48 were kanamycin resistant and had acquired the proline dehydrogenase defect of the donor strain, whereas the remaining 48 were kanamycin sensitive and could utilize L-proline. Of 39 transductants from strain JT34, 16 were kanamycin resistant and had acquired the proline transport defect of the donor strain, whereas 23 were kanamycin sensitive and could utilize L-proline. These linkages with *pyrC* (50 and 41%, respectively) are in agreement with those previously determined for the *put* genes (12, 19). In a subsequent study, derivatives of strains JT31 and JT34 have been prepared, and their reversion to *put*⁺ has been analyzed (20). Those derivatives revert to *put*⁺ at frequencies of approximately 10⁻⁷ with 100% coreversion to

kanamycin sensitivity. It is likely, therefore, that strains JT31 and JT34 each bear only a single copy of Tn5.

Biochemical characteristics of proline utilization mutants. The identity of the proline utilization defects of selected mutants has been confirmed by measuring their proline and glutamine transport and their proline dehydrogenase activities (Table 4). The bacteria were grown in minimal medium containing succinate and glycyl-L-proline. The latter is a weak inducer of the *put* genes which enters the bacteria via a peptide transport system, avoiding the inducer exclusion that occurs in *putP* mutant strains (19). As controls, the corresponding activities of an uncharacterized Tn5 insertion strain (JT27) and of a spontaneous AC^r mutant (WG78), each with apparently normal proline utilization capacity, have also been determined. Each putative *putP* mutation caused a 90 to 98% reduction in proline uptake activity without altering the glutamine uptake rate. The residual proline uptake activity is probably due to the second proline transport system, PP-II. The *putPA* deletion and *putA*::Tn5 insertion strains have negligible proline dehydrogenase activity. Strain JT31 has unusually high proline uptake activity; presumably it lacks the *putA* gene product. All of the characteristics initially detected by qualitative tests are therefore substantiated by these biochemical assays.

Substrate specificity of proline dehydrogenase. The proline dehydrogenases of both *E. coli* (13) and *S. typhimurium* (Menzel, Ph.D. thesis) have been purified and characterized. AC

TABLE 4. Biochemical characteristics of selected mutant strains^a

Strain	Relevant genotype	nmol min ⁻¹ mg of protein ⁻¹		
		Transport activity		Proline dehydrogenase
		Proline	Glutamine	
CSH4	<i>put</i> ⁺	5.4	2.3	15
WG78	<i>put</i> ⁺	4.7	2.0	9
WG69	<i>putP5</i>	0.2	1.8	12
RM2	Δ (<i>putPA</i>)100	0.6	2.0	<0.1
WG42	<i>putP4</i>	0.1	2.1	11
JT27	<i>put</i> ⁺ (Tn5)	7.0	2.1	10
JT31	<i>putA1</i> ::Tn5	63.0	1.8	<0.1
JT34	<i>PutP3</i> ::Tn5	0.2	2.1	14

^a Bacteria were grown on MOPS minimal medium containing succinate, NH₄Cl, and glycyl L-proline (200 μ g/ml). Amino acid uptake and proline dehydrogenase activities were measured as previously described. The substrate concentrations in the assays were 20 μ M proline and 10 μ M glutamine for transport and 0.4 M proline for proline dehydrogenase.

is a weak inhibitor (13), but not a substrate (Menzel, Ph.D. thesis), for proline dehydrogenase; the interaction of the enzyme with DHP has not been described before.

The substrate specificity of proline dehydrogenase has been investigated by using inverted membrane vesicles from strains CSH4 (*putP⁺ putA⁺*) and JT31 (*putP⁺ putA1::Tn5*). The membranes were prepared from bacteria grown in media containing succinate as carbon source, with L-proline present to fully induce expression of the *put* genes. Proline dehydrogenase activity (with proline as substrate) can be measured either very specifically via the reaction of oAB with Δ^1 -pyrroline carboxylic acid (the oAB assay) or more generally via the reduction of DCPIP. The two assays yielded proline oxidation rates of 25 ± 2 and 21 ± 1 nmol min⁻¹ mg of protein⁻¹, respectively, when six replicate assays of each type were completed, using a single membrane preparation for strain CSH4. No proline oxidation activity was detectable in membranes from strain JT31 by either assay. The DCPIP assay is, therefore, an adequate measure of proline dehydrogenase activity.

Initial attempts to detect DHP dehydrogenase activity with the oAB assay were unsuccessful. The DCPIP assay was therefore used to measure the rates of oxidation of L-proline, DHP, and AC by membranes from strains CSH4 and JT31 (Table 5). The rate of succinate oxidation was measured via the same assay system as a control. No AC oxidation activity was detectable, in agreement with previous results. The rate of DHP oxidation by membranes from strain CSH4 was three times greater than that of proline oxidation, and, surprisingly, membranes from strain JT31 showed significant DHP dehydrogenase activity. If the DHP oxidation rates are expressed as a fraction of the succinate dehydrogenase activity of the same preparation, then the DHP dehydrogenase activity of strain JT31 is 28% of that of strain CSH4. Since the dehy-

droproline used in these experiments was a mixture of the D- and L-isomers, the substrate activity of D,L-proline was also tested with membranes from strain JT31. No D-proline oxidation activity was detectable. These data suggest that DHP is a substrate for proline dehydrogenase, although its oxidation product does not form a colored complex with oAB. They also imply that DHP is oxidized by a second, membrane-associated dehydrogenase in *E. coli*.

DISCUSSION

The data presented here and elsewhere (20) support the view that the genetics and biochemistry of proline utilization in *E. coli* are closely analogous to the properties of the corresponding system in *S. typhimurium* (see above). Although the proline dehydrogenase of *S. typhimurium* also oxidizes Δ^1 -pyrroline carboxylate to glutamate (Menzel, Ph.D. thesis), no such activity has been detected in the purified *E. coli* enzyme (13). In view of the overall similarity between the two organisms reported here, a reexamination of the enzyme from *E. coli* is suggested.

The genetic data presented here show that proline dehydrogenase influences the sensitivity of *E. coli* strains to the toxic proline analog DHP (Table 4). They imply that DHP is oxidized, and thereby detoxified, by proline dehydrogenase. Direct biochemical assay shows that DHP is indeed an effective substrate for proline dehydrogenase, but that it is also oxidized by another membrane-associated dehydrogenase in *E. coli* (Table 5). The impact of these two oxidative enzymes on the cytotoxicity of DHP will depend on their relative kinetic characteristics and on the toxicity of their oxidation products. The characteristics of the membrane-associated proline dehydrogenase are being examined further.

These observations provide the basis for a number of mutant selection and screening procedures. The *putA* gene product is the repressor controlling *putP* and *putA* expression. Three classes of mutants mapping in *putA* can therefore be identified: mutants lacking proline dehydrogenase activity and constitutive for proline transport, mutants lacking proline dehydrogenase activity but with normally regulated proline transport, and mutants with normal proline dehydrogenase activity that are constitutive for both proline dehydrogenase and proline transport (Menzel, Ph.D. thesis). These three classes of *putA* mutants should yield the analog resistance phenotypes AC^{ss} DHP^{ss}, AC⁺ DHP^{ss}, and AC^{ss} DHP⁺, respectively. The first of these phenotypes has been observed as that of the *putA::Tn5* insertion mutants identified during this study, and the last has been observed, albeit

TABLE 5. Substrate specificity of proline dehydrogenase^a

Substrate	Enzyme activity (nmol min ⁻¹ mg of protein ⁻¹)	
	CSH4	JT31
L-Proline	21 (1)	ND
AC	ND	ND
DHP	64 (7)	20 (1)
Succinate	75 (7)	85 (15)

^a Enzyme activities were measured by DCPIP reduction as described in the text. Values in parentheses are standard deviations. Each substrate was present at a 60 mM concentration. ND, not detectable.

artificially, as the phenotype of *E. coli* strains bearing multicopy hybrid plasmids containing the *put* genes (20). DHP probably enters *putP* strains via the second proline transport system, PP-II, that requires an intact *proP* gene (1, 8). We are therefore attempting to select *proP* mutants from *putP putA* strains of *E. coli* and *S. typhimurium* by DHP resistance. No other positive selection for *proP* mutants has yet been devised since a readily detectable phenotype for *proP* mutants has been illustrated only in strains that are proline auxotrophic, requiring PP-II activity to meet that requirement.

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