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Mutants of *Salmonella typhimurium* That Are Insensitive to Catabolite Repression of Proline Degradation

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In *Salmonella typhimurium* the two enzymes of proline catabolism, proline oxidase and Δ^1-pyrroline-5-carboxylic acid dehydrogenase, are subject to catabolite repression when the cells are grown in the presence of glucose. Mutants partially relieved of catabolite repression (PutR) for the proline catabolic enzymes have been isolated by selection on agar plates containing glucose and proline. The specificity of the catabolite repression-insensitive character for the enzymes of proline utilization has been confirmed by an analysis of other unrelated catabolic enzymes. Histidase and amylomaltase of the mutant strains are equally as sensitive to glucose repression as are the enzymes from the wild type. All four PutR mutants exhibit higher induced and higher basal levels of proline oxidase as compared with the corresponding wild-type levels. The mutations of three strains tested are cotransducible with constitutive, pleiotrophic-negative and structural gene mutations of the *put* region. Three-factor crosses indicate that two *putR* mutations are located at one end of the cluster of *put* mutations.

The study of catabolite repression-insensitive mutants of the proline catabolic pathway in *Salmonella typhimurium* was undertaken primarily to continue the elucidation of the molecular means of regulation of the synthesis of the proline catabolic enzymes. The two enzymes of the pathway that convert proline to glutamate are proline oxidase (L-proline:O_2 oxidoreductase, EC 1.4.3.2) and P5C dehydrogenase (Δ^1-pyrroline-5-carboxylate:nicotinamide adenine dinucleotide phosphate [NADP] oxidoreductase, EC 1.5.1.2) (8, 9). Proline oxidase catalyzes the conversion of proline to the intermediate, L-Δ^1-pyrroline-5-carboxylic acid (P5C); P5C dehydrogenase catalyzes the conversion of P5C to glutamate.

It was also of interest to compare the regulation of proline degradation to that of histidine degradation in this organism (4, 15-17) because both pathways yield glutamate which can be utilized as a sole nitrogen source.

MATERIALS AND METHODS

Chemicals. L-Proline, L-histidine, and the disodium salt of D-glucose-6-phosphate (G-6-P) were obtained from the Sigma Chemical Co. Eastman Organic Chemicals supplied ethylmethanesulfonate and hexadecyltrimethyl ammonium bromide. Glucostat reagent was purchased from Worthington Biochemical Corp., *o*-aminobenzaldehyde from K and K Laboratories, and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine from Aldrich Chemical Co.

Bacterial strains. The strains listed in Table 1 were derived from *S. typhimurium* strain 15-59. Because the genes for the proline utilization system have not been identified, the mutant strains are designated by phenotype rather than by genotype. According to the recommendations by Demerec et al. (7), the following symbols have been devised for the proline utilization system: PutA, proline oxidase-negative; PutB, P5C dehydrogenase-negative; PutP, pleiotrophic-negative for both enzymes; PutC, constitutive for both enzymes; PutR, insensitivity of both enzymes to catabolite repression. A plus sign signifies the wild-type phenotype; a minus sign signifies a general mutant phenotype, whereas a number signifies a specific mutant strain.

Cultivation of bacteria. The medium used is medium E of Vogel and Bonner, except for the lack of citrate and the addition of trace elements (1). To the salt base were added nitrogen and carbon sources, which had been sterilized separately. They included 0.2% ammonium sulfate and 0.2% sodium succinate. Specific supplementation with 0.2% proline, 0.2% histidine, 0.2% maltose, and 0.4% glucose depended on the experiment. For the transductions, cells were cultivated in LB broth, a rich medium (3).

TABLE 1. *Strains of Salmonella typhimurium*

Strain	Mutation designation	Derivation
AR	(Wild-type)	B. Magasanik, NE (4)
AR17	*putP23*	S. Dendinger (8)
AR24[a]	*R111*	B. Magasanik, NE1 (4)
AR25[b]	*hutR9*	B. Magasanik, NE2 (4)
AR27	*putA26*	S. Dendinger (8)
AR63	*putC1*	S. Dendinger (8)
AR65	*putC3*	S. Dendinger (8)
AR71	*putC1,B58*	S. Dendinger (8)
AR74	*putC3,P61*	S. Dendinger (8)
AR111	*putC3,B86*	S. Dendinger (8)
AR167	*putR7*	EMS[c] of AR
AR172	*putR2*	EMS of AR
AR173	*putR3*	EMS of AR
AR174	*putR4*	EMS of AR
AR182	*putC3,P108*	S. Dendinger (8)

[a] Phenotype of mutant is general insensitivity to catabolite repression (4).

[b] Phenotype of mutant is catabolite-insensitive synthesis of histidase and urocanase (4).

[c] Ethylmethanesulfonate.

Fifteen-milliliter cultures were grown at 37 C in 125-ml Erlenmeyer side arm flasks with aeration. A Klett-Summerson photoelectric colorimeter with a no. 42 filter was used to measure growth. The growth measurements were correlated to cell numbers by a standard curve.

Most agar plates were prepared by adding a 3% solution of agar to an equal volume of the corresponding double strength liquid medium. For the G-6-P-proline plates, however, G-6-P was sterilized by membrane filtration (Millipore Corp.) and added to purified agar to a final concentration of 0.4%. LB agar and LB soft agar plates contained 1.5 and 0.75% agar, respectively.

Isolation of mutant strains. Mutangenesis involved adding a drop of ethylmethanesulfonate (EMS) to the center of an agar plate on which 4×10^7 cells had previously been spread. Mutants resistant to catabolite repression were selected on agar plates containing glucose as the carbon source and proline as the only nitrogen source. The purified isolates were required to pass three screening tests before they were designated at PutR, that is, a mutant in which only the enzymes of proline degradation are resistant to catabolite repression. Better growth of streaks on glucose-proline plates than that exhibited by the wild type was required as well as good growth on glucose-ammonia plates and poor growth on glucose-histidine plates.

Enzyme assays. For the following four assays, a 0.5-ml sample of an overnight culture in minimal medium supplemented with ammonium sulfate and sodium succinate was added to 15 ml of the appropriate medium, and the flasks were incubated on a rotary shaker at 37 C until the cell population reached between 5.2×10^8 and 6.8×10^8 cells per ml.

Proline oxidase and P5C dehydrogenase were assayed by the methods of Dendinger and Brill (8). The method of Lowry et al. (10) was used to determine protein concentration. The specific activity of proline oxidase is expressed as micromoles of P5C formed per minute per 10^{11} cells; that of P5C dehydrogenase is expressed as micromoles of reduced NADP formed per minute per milligram of protein.

The histidase (histidine ammonia lyase, EC 4.3.1.3) assay as reported by Brill and Magasanik (4) was modified to a whole-cell assay. Culture samples of 1.5 ml were centrifuged at 23,000 $\times$ *g* for 10 min at 4 C. The pellet was washed with 3.0 ml of 0.2 M diethanolamine (Cl^-) at *p*H 9.4. After a second centrifugation, the cells were suspended in 1.5 ml of the diethanolamine buffer, to which 0.15 ml of a hexadecyltrimethyl ammonium bromide-azide mixture (1 mg/ml in 0.1 M NaN_3) was added. After 5 min, an 0.08-ml cell sample was added to the following reaction mixture: 0.50 ml of diethanolamine buffer, 0.20 ml of 0.1 M L-histidine solution, and 0.52 ml of water. After an incubation period of 45 min at 30 C the reaction was stopped with 0.1 ml of 35% perchloric acid. The tubes were centifuged to remove the precipitated protein. In 20 min, the absorbance at 268 nm was measured on a Gilford 2400 recording spectrophotometer. The values were corrected by subtracting the absorbance of an extract blank containing no histidine. The specific activity of histidase is expressed as micromoles of urocanic acid formed per minute per 10^{13} cells.

The assay as described by Brill and Magasanik (4) was used to determine amylomaltase (maltose-4-glucosyltransferase, EC 2.4.1.3) activity. For the preparation of the cell extract, a 15-ml culture was grown to a titer of about 6.0×10^8 cells per ml. The cells were harvested by centrifugation. After a single wash with 0.01 M potassium phosphate buffer at *p*H 7.0, the cells were suspended in 1.0 ml of buffer. The supernatant solution used as the cell extract was prepared by a 30-sec period of ultrasonic treatment in a Branson sonic oscillator, followed by centrifugation at 27,000 $\times$ *g* for 15 min. The specific activity of amylomaltase is expressed as nanomoles of glucose formed per minute per milligram of protein.

Spot test for constitutivity. Colonies to be tested for constitutivity of proline oxidase were streaked onto a succinate-ammonia plate. Constitutive colonies were recognized by the yellow color of a P5C-*o*-aminobenzaldehyde complex. The spot test for constitutivity has been described by Dendinger and Brill (8).

Preparation of phage lysates. Phage lysates of P376 were prepared by the method of Meiss, Brill, and Magasanik (12).

Transductions. In all transductions a Put^- strain served as the recipient and phage lysates of PutR strains served as the donor. The recipient strain was grown in LB broth to a titer of 2.7×10^8 cells per ml. The transduction mixture, consisting of 4 ml of a culture of the recipient strain and phage added to a multiplicity of 5, was incubated at 37 C for 10 min. After centrifugation, the cells were washed once with 4 ml of minimal medium. The final suspension of the pellet was in a volume of 1.0 ml. Samples of 0.1 ml of the undiluted suspension, a 1:10 dilution, and a 1:100 dilution were plated on succinate-proline plates. The selection was for the ability to utilize

proline as the only nitrogen source. Phage and bacterial controls were included to test for contamination and reversion. Transductant colonies were picked and streaked onto succinate-proline plates to enrich for the transductant. The constitutive or inducible character of the transductants was determined by the spot test for constitutivity. The catabolite repression character was determined by observing the growth of a streak on G-6-P-proline plates.

RESULTS

Growth characteristics of the PutR strains. The mean doubling times during exponential growth of several PutR mutants in glucose-ammonia, glucose-proline, and glucose-histidine liquid media are reported in Table 2. Minimal medium containing 0.4% glucose was supplemented with 0.2% of the nitrogen source. Strains AR24 and AR25, isolated previously by Brill and Magasanik (4), are two controls that have been included. Strain AR24 has an alteration in glucose metabolism that confers general resistance to catabolite repression. Strain AR25, designated as *hutR*, is a catabolite repression-insensitive mutant that is specific for the urocanase-histidase system. Strains AR167, AR172, AR173, and AR174 are four PutR strains isolated from a culture mutagenized with ethylmethanesulfonate. All of the specific catabolite repression-insensitive mutants, including AR25, have the same doubling times essentially in glucose-ammonia medium as does the wild type. On the other hand, strain AR24, the general catabolite repression-insensitive mutant strain, exhibits a longer mean doubling time. The doubling times in glucose-proline medium for the wild type and strain AR25 are nearly identical and much longer than the doubling times of the strains whose mutations affect catabolite repression of the Put system, whether specifically (AR167, AR172, AR173, and AR174) or generally (AR24). In glucose-histidine medium, the doubling times for all the strains in which the mutations affect catabolite repression of the *hut* system, whether specifically (AR25) or generally (AR24), are significantly shorter than those of the wild type or the PutR strains. For some reason that we are unable to explain, strains AR167, AR172, and AR174 grow more slowly than the wild type in glucose-histidine medium.

Proline oxidase characterization of the PutR strains. The levels of proline oxidase of the wild type and several mutant strains under uninduced, induced, and catabolite-repressed conditions are given in Table 3. The basic growth medium contains 0.2% ammonium sulfate and 0.2% sodium succinate. In the presence of the inducer, proline, the wild-type level of proline oxidase is about 25-fold higher than it is in the absence of the inducer. The catabolite-repressed level of wild-type proline oxidase as measured in the presence of glucose is 22% that of the induced level. The proline oxidase of strain AR25, containing a mutation that confers resistance to catabolite repression specifically for the *hut* system, is catabolite-repressed to the same extent that the wild type is repressed. The other control, strain AR24, the general catabolite-insensitive mutant strain, is almost completely resistant to catabolite repression for proline oxidase. The partially constitutive mutant AR63 exhibits catabolite repression of proline oxidase identical to that of wild type. The fully constitutive mutant AR65 exhibits a specific activity on medium containing proline and glucose that is slightly higher than that of wild type, but it exhibits also a hyperinduced level. Because the catabolite-repressed level (0.65) is still only 20% of the hyperinduced level (3.2), strain

TABLE 2. *Mean doubling time (hours) of wild-type and mutant strains*[a]

Strain	Glucose-ammonia[b]	Glucose-proline[b]	Glucose-histidine[b]
AR (wild-type)	0.9	6.3	13
AR24	1.5	1.4	1.3
AR25	0.9	6.1	6.0
AR167	1.0	3.7	21
AR172	0.9	3.6	20
AR173	1.0	5.0	13
AR174	1.0	2.6	20

[a] A 10% error was observed in the reproducibility of the doubling times.

[b] Carbon and nitrogen source in minimal medium.

TABLE 3. *Specific activities of proline oxidase*

Strain	Specific activity with additions to minimal medium			
	None	Proline	Proline and glucose	
AR (wild-type)	0.10	2.5	0.55	(22%)[a]
AR24	0.14	2.4	2.2	(92%)
AR25		2.2	0.54	(24%)
AR63	0.92	2.4	0.55	(23%)
AR65	4.7	3.2	0.65	(20%)
AR167	0.19	3.3	1.0	(30%)
AR172	0.34	3.8	1.8	(47%)
AR173	0.30	3.7	1.8	(49%)
AR174	0.13	4.3	1.4	(33%)

[a] Percentages listed are a ratio of the catabolite-repressed level to the corresponding induced level. The average wild-type induced level for 18 assays is 2.5 with a standard deviation of ±0.2.

AR65 is designated as a constitutive mutant that is fully sensitive to catabolite repression. Therefore, mutants constitutive for the Put enzymes retain their sensitivity to catabolite repression. This observation is evidence that the decrease in enzyme level upon addition of glucose is not due to competition for proline uptake by glucose. One would predict that cyclic adenosine monophosphate can reverse this repression.

The remaining mutants are partially resistant to catabolite repression; their levels of proline oxidase in the presence of glucose are about two to three times higher than the wild-type level. No mutant strain was isolated that was completely resistant to catabolite repression for the Put system alone. The basal levels and the induced levels of the PutR strains are higher than the corresponding levels of the wild type. For strain AR167, the catabolite-repressed specific activity of proline oxidase is 0.30 of the induced level (3.3); for strains AR172 and AR173, the catabolite-repressed level is nearly 0.50 of the induced level (3.8); for strain AR174, the catabolite-repressed level is 0.33 of the induced level (4.3). Five mutant strains not reported exhibit proline oxidase levels identical to those observed in strain AR167. In spite of the hyperinduced levels exhibited by the PutR strains, the repression ratio of all the mutants (catabolite-repressed level divided by induced level) is greater than the repression ratio of the wild type, 0.22.

P5C dehydrogenase characterization of the PutR strains. Possible pleiotropic effects of the PutR mutations were investigated by determining the levels of P5C dehydrogenase, the second enzyme of the proline catabolic pathway. The P5C dehydrogenase of the PutR strains is simultaneously partially relieved of catabolite repression and is hyperinducible (Table 4). Consequently, in a qualitative sense, the two enzymes of the proline catabolic pathway in the PutR strains respond to changes in induction and catabolite repression in a coordinate fashion. Quantitatively, however, these changes are not precisely the same for both enzymes. P5C dehydrogenase activity in the PutR strains seems to be less relieved of catabolite repression than proline oxidase activity.

TABLE 4. *Specific activities of P5C dehydrogenase*

Strain	Specific activity with additions to minimal medium	
	Proline	Proline and glucose
AR (wild-type)	11	3 (27%)[a]
AR167	39	13 (33%)
AR172	52	21 (40%)
AR173	44	19 (43%)
AR174	53	15 (28%)

[a] Percentages listed are a ratio of the catabolite-repressed level to the corresponding induced level.

Unrelated catabolic enzyme analysis of the PutR strains. The sensitivity of the enzymes histidase and amylomaltase to catabolite repression was used as a criterion of the specificity of the effects of the PutR mutations. Histidase and amylomaltase were assayed in the presence of their respective inducers and in the presence of both inducer and glucose. As recorded in Table 5, the catabolite-repressed level of the wild-type histidase is 15% of the induced level. Considerable relief of repression is evident in strain AR24, the general catabolite repression-insensitive mutant, by a catabolite-repressed level that is 78% that of the induced level. Similar relief is exhibited by the catabolite-insensitive mutant strain specific for the *hut* system, AR25, along with a hyperinduced level that is twofold higher than the wild-type induced level. In none of the four PutR strains is the catabolite-repressed level, expressed as a percentage of the corresponding induced level, higher than that of the wild type. It can be concluded that the histidase enzymes of the PutR strains AR167, AR172, AR173, and AR174, are fully sensitive to catabolite repression by glucose. Furthermore, the amylomaltase enzymes of the PutR strains are fully sensitive to catabolite repression by glucose (Table 6).

Linkage of the mutations of strains AR172 (putR2) and AR173 (putR3) to the put region. The linkage relationship of *putR2* and

TABLE 5. *Specific activities of histidase*

Strain	Specific activity with additions to minimal medium	
	Histidine	Histidine and glucose
AR (wild-type)	113	17 (15%)[a]
AR24	124	97 (78%)
AR25	225	101 (45%)
AR167	120	17 (14%)
AR172	116	17 (15%)
AR173	103	8 (8%)
AR174	141	19 (13%)

[a] Percentages listed are a ratio of the catabolite-repressed level to the corresponding induced level. For 14 assays, the average wild-type induced level is 113 with a standard deviation of ±20.

putR3 to other *put*⁻ mutations has been established by transductions, in which a phage lysate of strain AR172 or strain AR173 served as the donor and a Put⁻ strain served as the recipient (Table 7). Recombinants were selected for their ability to grow on proline as the only nitrogen source.

Just as the enzyme data for strains AR172 and AR173 are nearly identical, so are the cotransduction data nearly identical. The cotransduction values ranging from 80 to 100% indicate that in any cross, at least 80% of the transductants inherited the PutR⁻ character along with the Put⁺ character. Close linkage of *putR2* and *putR3* to the three pleiotropic-negative mutations, *putP108*, *putP61*, and *putP23*, is greater than 92% as shown by crosses 1 to 5. *putR3* is 90% contransduced with *putA26* (cross 6). *putR2* and *putR3* are about 80% cotransduced with both *putB*⁻ mutations, *B86* and *B58* (crosses 7 to 10). In summary, the *putR* mutations are linked to P5C dehydrogenase-negative mutations, proline oxidase-negative mutations, and pleiotrophic-negative mutations with respectively closer linkage.

TABLE 6. *Specific activities of amylomaltase*[a]

Strain	Specific activity with additions to minimal medium	
	Maltose	Maltose and glucose
AR (wild-type)	243	32 (13%)
AR24	237	211 (89%)
AR167	230	10 (4%)
AR172	199	10 (5%)
AR173	239	10 (4%)
AR174	278	11 (4%)

[a] For 11 assays, the average wild-type induced level is 243 with a standard deviation of ±18.

Linkage of the mutation of strain AR174 (putR4) to the put region. When similar crosses are performed using strain AR174 as the donor, the resulting cotransduction value of *putR4* and other *put*⁻ mutations is never more than 25%. All the mutations of the Put⁻ mutants isolated thus far are at least 65% cotransduced with respect to each other (8). In light of this result, one interpretation of the results with *putR4* is that the *putR4* mutation is closely linked to the *put* region, but that some kind of interference inherent in the nature of the *putR4* mutation favors a crossover between *putR4* and *put*⁻ mutations. Another interpretation is that the mutation *putR4* is located in a regulatory locus of the *put* region that is close but not part of the main cluster. Because of the unique nature of strain AR174, the first interpretation is preferred at present.

Order of putR2 and putR3 with respect to other put mutations. In regard to a pleiotropic-negative mutation, a proline oxidase-negative mutation, and P5C dehydrogenase-negative mutation, Dendinger and Brill have established an order of *P61-C3-A26-B58* (8). The three-factor crosses in Table 8 suggest an order of *putR2* and *putR3* with respect to these mutations. In crosses 1 and 2, phage lysates of strains AR172 and AR173 were used as donors, and AR74, containing a pleiotropic-negative mutation (*P61*) and a constitutive mutation (*C3*), was used as the recipient. The selected

TABLE 7. *Cotransduction of putR2 and putR3 with other put⁻ mutations*[a]

Cross	Donor		Recipient		Selected phenotype	Percentage inheritance of unselected PutR⁻ phenotype
	Strain	Character	Strain	Character		
1	AR172	*R2,P⁺,C⁺*	AR182	*R⁺,P108,C3*	PutP⁺	100
2	AR173	*R3,P⁺,C⁺*	AR182	*R⁺,P108,C3*	PutP⁺	100
3	AR172	*R2,P⁺,C⁺*	AR74	*R⁺,P61,C3*	PutP⁺	96
4	AR173	*R3,P⁺,C⁺*	AR74	*R⁺,P61,C3*	PutP⁺	96
5	AR172	*R2,P⁺,C⁺*	AR17	*R⁺,P23,C⁺*	PutP⁺	93
6	AR173	*R3,A⁺,C⁺*	AR27	*R⁺,A26,C⁺*	PutA⁺	90
7	AR172	*R2,B⁺,C⁺*	AR111	*R⁺,B86,C3*	PutB⁺	84
8	AR173	*R3,B⁺,C⁺*	AR111	*R⁺,B86,C3*	PutB⁺	83
9	AR172	*R2,B⁺,C⁺*	AR71	*R⁺B58,C1*	PutB⁺	80[b]
10	AR173	*R3,B⁺,C⁺*	AR71	*R⁺,B58,C1*	PutB⁺	80[b]

[a] In all genetic crosses, the *put*⁺ character was transduced into a *put*⁻ strain by a phase lysate of a putR mutant. For each transduction, 144 transductants were picked and streaked onto succinate-proline plates. The catabolite repression phenotype was determined by growth on glucose-6-phosphate-proline plates. The cotransduction values are expressed as the percentage of transductants inheriting the donor putR phenotype.

[b] The percentage could be higher because the recipient AR71 reverts readily.

TABLE 8. *Order of putR2 and putR3 with respect to putP61, putP108, and putC3*[a]

Cross	Donor		Recipient		Selected phenotype	No. of unselected recombinants			
	Strain	Character	Strain	Character		R^-,C^+	R^+,C^+	R^-,C^-	R^+,C^-
1	AR172	*R2*	AR74	*P61,C3*	$PutP^+$	273	5	5	4
2	AR173	*R3*	AR74	*P61,C3*	$PutP^+$	276	2	6	4
3	AR172	*R2*	AR182	*P108,C3*	$PutP^+$	130	0	14	0
4	AR173	*R3*	AR182	*P108,C3*	$PutP^+$	112	0	14	0

[a] In each transduction, the selected phenotype was Put^+, the ability to grow with proline as the only nitrogen source. The unselected characters of constitutivity and catabolite-insensitivity were determined by the spot test for constitutivity and growth on glucose-6-phosphate-proline plates. The numbers of transductants of the four possible classes are listed.

character was Put^+. The unselected characters of constitutivity and resistance to catabolite repression were determined. The results of crosses 1 and 2 are interpreted on the assumption that a quadruple crossover event will be rare in comparision with a double crossover event. Representatives of all four possible classes were found in both crosses. Instead of one rare class, three classes are almost equally as rare. Lack of a single rare class indicates that no quadruple crossovers were required to generate the four classes. Thus the tentative order is *R2, R3-P61-C3*.

In crosses 3 and 4, strain AR182, containing a pleiotropic-negative mutation (*P108*) and a constitutive mutation (*C3*), was used as the recipient. The location of *P108* has not been determined with respect to other *put* mutations, but recombination between *P108* and *P61* and between *P108* and *C3* has been shown (S. Dendinger, Ph.D. thesis, Univ. of Wisconsin, 1971). All of the 144 transductants in cross 3 exhibit the $PutR^-$ phenotype; similarly, all of the 126 transductants in cross 4 exhibit the $PutR^-$ phenotype. This lack of the $PutR^+$ character among the transductants reveals that no crossing over occurred between the pleiotropic-negative mutation, *P108*, and the *putR* mutations. In conjunction with Dendinger's results and the order stated above, the proposed order of seven *put* mutations is *P108, R2, R3-P61-C3-A26-B58*. The order of *P108, R2*, and *R3* with respect to each other is unknown.

DISCUSSION

The results indicate that the proline oxidase and P5C dehydrogenase of the four PutR strains are partially resistant to catabolite repression by glucose, whereas the histidase and amylomaltase of the PutR strains remain just as sensitive to glucose repression as the wild-type enzymes. These results provide a biochemical demonstration that the catabolite repression insensitivity of strains AR167, AR172, AR173, and AR174 is specific for the proline catabolic system. The genetic determination of the close linkage of the mutations of strains AR172 (*putR2*) and AR173 (*putR3*) to the *put* region confirms the specificity of the catabolite insensitivity of these two strains. The 25% cotransduction linkage of the mutation of strain AR174 (PutR4) makes the genetic determination of specificity less clear for this mutant. Difficulties in the recognition of the $PutR^-$ phenotype of strain AR167 on agar plates have made the determination of the linkage of the mutation of AR167 to the *put* region impossible at this time.

The similarities between the PutR strains and specific catabolite-insensitive strains reported for other catabolic systems are unmistakable (2, 4–6, 11, 12). The types of catabolite-insensitive mutant strains reported are either of an R or a Q phenotype. R signifies inducibility and insensitivity to catabolite repression; Q signifies constitutivity and insensitivity to catabolite repression. For the *lac* system, the catabolite-insensitive mutant strains are of the R phenotype (6, 14). Both R and Q phenotypes have been reported in mutants of the *hut* system in *S. typhimurium* and *B. subtilis* (4, 5). Only Q types have been reported for the D-serine deaminase system (11) and the glycerol utilization system (2). Among the R-type strains, some exhibit hyperinducibility; specifically, they are the strains carrying the *hutR* mutations in *S. typhimurium* (4) and the promoter mutation in *E. coli*. (6). Biochemically, the PutR mutants are most similar to these hyperinducible R type strains. Just as the phenotype of the strains harboring mutations that effect catabolite insensitivity and hyperinducibility of the *hut* system and the *lac* system can be ascribed to up-promoter mutations, so would the authors like to ascribe the phenotypes of the PutR strains to up-promoter mutations.

Hypotheses regarding the nature of the catabolite-sensitive site in the proline oxidase-P5C dehydrogenase system require an elucidation of the *put* region as well as a map location for the *putR* mutations. Unfortunately, the operator, repressor, or activator gene, and structural gene roles cannot be assigned as yet to the various mutations in the *put* region (8). However, that the structural genes for proline oxidase and P5C dehydrogenase are part of the same operon is suggested by constitutive and pleiotropic-negative mutations that affect both enzyme activities to the same degree (8). Because the selection of these mutants was not specific for proline oxidase, but rather required that the mutants grow well on proline in the presence of glucose, a mutation that specifically causes either proline oxidase or P5C dehydrogenase activity to escape repression might not be detected by our selection techniques. Preliminary mapping experiments suggest that the *R2* and *R3* mutations of strains AR172 and AR173, respectively, are located at one end of the cluster of *put* mutations that define the *put* region on the chromosome.

More than likely, the *R2* and *R3* mutations define a promoter site of the *put* operon. Accordingly, *R2* and *R3* are up-promoter mutations that simultaneously render the *put* operon partially insensitive to catabolite repression. The first criterion of a promoter as defined by Scaife and Beckwith (13) is the pleiotropic effect on operon expression. The reported results satisfy this criterion; both proline oxidase and P5C dehydrogenase of the *putR* strains are hyperinducible and partially resistant to catabolite repression.

Other types of mutations, however, could explain the PutR phenotypes. One explanation is that a duplication of the *put* region has occurred either in the form of an actual duplication on the chromosome or in the form of an extrachromosomal element bearing the *put* region. The occurrence of such duplications would be expected to be so rare that such an explanation might account for one PutR mutant but certainly not for the nine PutR mutants that have been isolated.

Based on the assumption that a repressor exists for the Put region that is similar in function to the *lac* repressor, yet another possibility is that the PutR phenotype is due to a repressor mutation. For example, the repressor mutation might result in tighter binding between the inducer and the repressor molecules such that the action of the inducer becomes more effective than normal, that is, more repressor is removed from the operator site. Thus, a repressor mutation as well as a promoter mutation would account for the increased level of expression of the *put* operon. The observation that the PutR mutants are partially relieved of catabolite repression as revealed by higher repression ratios (catabolite-repressed enzyme level divided by induced enzyme level) for proline oxidase and P5C dehydrogenase than the corresponding wild-type repression ratios, however, indicates that catabolite insensitivity and hyperinducibility have been effected simultaneously. Since no repressor mutation has been reported that causes a change in the catabolite repression character of an operon, the promoter remains the most likely candidate for the site of the PutR mutations. The final designation of the PutR mutations as promoter mutations awaits the establishment of *cis* dominance and the distinction from repressor-operator functions and sites.

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