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Regulation of the Genes for Proline Utilization in *Salmonella typhimurium*: Autogenous Repression by the *putA* Gene Product

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Two genes are involved in the degradation of proline to glutamic acid. The *putP* gene encodes a proline permease; the *putA* gene encodes a bifunctional degradative enzyme. Although the two genes are transcribed independently, both genes are induced by exogenous proline and are subject to catabolite repression. The *putA* gene product appears to function also as an autogenous repressor protein acting on both the *putA* and *putP* transcription units. Regulatory mutations map throughout the *putA* gene; these are correctable by an episomal $putA^+$ gene, which acts *in trans* to restore repression.

1. Introduction

Mutants unable to utilize proline as a sole nitrogen source define a cluster of genes (*put*). The *put* gene cluster is located between the *pyrC* and *pyrD* loci at minute 22 of the *Salmonella* chromosome (Sanderson & Hartman, 1978). The *putA* gene encodes a bifunctional degradative enzyme that catalyses the oxidation of proline to pyrroline-5-carboxylic acid; the enzyme also possesses a dehydrogenase activity that converts pyrroline-5-carboxylic acid to glutamic acid. The *putP* gene encodes the cell's major proline permease (Ratzkin *et al.*, 1978; Ratzkin & Roth, 1978; Wood & Zadworny, 1979; Menzel, 1980).

The *put* mutants previously isolated and characterized were classified according to their ability to utilize leucyl-proline (Leu-Pro) and by their sensitivity to the proline analogue, azetidine carboxylic acid. The dipeptide Leu-Pro is transported into the cell and cleaved to its constituent amino acids by systems independent of the *put* genes. Proline, but not leucine, can be subsequently degraded by *Salmonella typhimurium* to provide metabolically useful carbon and nitrogen (Gutnick *et al.*, 1969). Therefore Put^- mutants that are also unable to utilize Leu-Pro must be defective in the proline-degradative activities. Virtually all Put^- mutations affecting the *putA* gene lack both enzymatic activities. AZ† is transported into the

† Abbreviations used: AZ, azetidine carboxylic acid; HA, hydroxylamine.

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cell by the proline transport system (Tristram & Neale, 1968) and is toxic by virtue of its incorporation into proteins in the place of proline (Fowden & Richmond, 1963). Mutants that are Put$^-$ and AZ-resistant are defective in proline transport.

Previous work has led to the conclusion that the *putA* and *putP* genes are transcribed independently and are subject to induction by exogenous proline and catabolite repression (Ratzkin & Roth, 1978; Dendinger & Brill, 1970).

In this paper we describe the isolation, mapping and complementation behavior of new Put$^-$ and constitutive mutations. Our analysis of these mutants leads us to the conclusion that the *putA* protein serves both as a bifunctional proline-degradative enzyme and also as a repressor of both the *putA* and *putP* genes.

2. Materials and Methods

(a) *Media and growth conditions*

Difco nutrient broth (0·8%) containing 0·5% (w/v) NaCl was used as a complex medium. Vogel & Bonner (1956) E medium containing 2% (w/v) glucose was used as minimal medium. Other carbon sources were used in the NCE medium of Berkowitz *et al.* (1968). Medium in which proline (0·2%) is the sole nitrogen source (PSN) has been described by Ratzkin & Roth (1978). Except as noted, amino acids are added as needed at approximately 0·3 mM. Adenine and uracil were used at 0·4 mM as needed. Tetracycline was added to complex media at 25 μg/ml and at 10 μg/ml to minimal media. Kanamycin was added to complex media at 50 μg/ml and at 75 μg/ml to minimal media. Solid media contained 1·5% Difco agar except for PSN medium, which was solidified by 1·0% highly purified agar (Difco Ionagar). Cells were grown at 37°C unless otherwise indicated. Liquid cultures were grown with gyratory shaking.

(b) put *indicator plates*

By modifying a medium described by Bochner & Savageau (1977), we developed a tetrazolium indicator medium on which Put$^+$ colonies are red and Put$^-$ colonies are white after 16 h growth at 37°C. The *put* indicator plates contain 5·6 g KH_2PO_4, 2·4 g K_2HPO_4, 2·0 g Bactopeptone, 1·6 g proline, 1 mM-$MgSO_4$, 0·0025% tetrazolium dye (Difco) and 15 g agar per liter. The KH_2PO_4, K_2HPO_4, Bactopeptone, proline and agar were autoclaved together. The $MgSO_4$ and tetrazolium were autoclaved individually and then added to the media subsequent to autoclaving. The addition of either tetracycline (10 μg/ml) or kanamycin (75 μg/ml) does not affect the behaviour of this medium. The presence of a mutation causing auxotrophy may interfere with the use of the indicator plate. Often auxotrophy will cause a Put$^+$ strain to be scored as Put$^-$ (white colonies). These mutations were screened out in obtaining the *put* mutants discussed in Results.

(c) *Leu-Pro test*

Strains to be tested for the ability of the dipeptide leucyl-proline to satisfy their nitrogen requirement were grown to stationary growth phase in nutrient broth and allowed to stand at room temperature for 12 to 24 h. This treatment eliminates residual growth seen on succinate-no-nitrogen plates. This test was performed on the PSN medium described by Ratzkin & Roth (1978), from which the proline has been omitted. Strains to be tested were streaked radially on the above medium from a sterile filter disk containing 20 μl 0·2 M-leucyl-proline. Growth was scored after 2 to 4 days. Put$^-$ strains defective in the *putA* gene failed to grow anywhere on the streak. Put$^+$ strains and Put$^-$ strains defective for the *putP* gene grew all along the streak with growth being more luxuriant nearer the filter disk. All tests were performed with control Put$^+$, *putA* and *putP* strains tested in parallel. Growth on succinate

was examined as a control on a plate in which the filter disk had been saturated with ammonium sulfate. The Leu-Pro test (for scoring the *putA*$^-$ genotype) was originally described by Ratzkin & Roth (1978).

(d) *AZ sensitivity tests*

Strains to be tested for their sensitivity to the proline analogue azetidine carboxylic acid were grown as in the Leu-Pro test. This test was performed on NCE minimal media supplemented with 0·4% sodium succinate. Strains to be tested were streaked radially on the above media from a sterile filter disk containing 20 μl 0·2 M-azetidine. The significance of the various levels of AZ sensitivity are discussed in the text. The AZ test was originally described by Ratzkin & Roth (1978). All tests were performed with control *put*$^+$, *putP*$^-$, *putA* (A$^+$C$^-$) *putA* (A$^-$C$^+$) mutants examined in parallel. Strains were scored as AZ^S if the radial streak was inhibited 12 to 16 mm from the filter disk. AZ^{SS} strains were inhibited 20 to 35 mm from the disk. AZ^R strains showed no inhibition.

(e) O-*aminobenzaldehyde test*

The use of *O*-aminobenzaldehyde to detect mutants constitutive for the expression of proline oxidase in a Petri dish assay was originally described by Dendinger & Brill (1970). In our tests colonies were replica-printed to glass Petri dishes containing solid NCE media supplemented with 0·4% sodium succinate. The colonies were permeabilized by placing 2 ml of toluene in the lid of the inverted Petri dish and incubating for 15 min at 37°C. At this time the toluene was removed and a filter disk of Whatman no. 1 filter paper saturated with a solution containing 3 M-proline, 5×10^{-2} M-*O*-aminobenzaldehyde, 0·2 M-cacodylic acid buffer (pH 6·8) and 0·01% chloramphenicol was placed gently over the colonies. The Petri dish was then incubated at 37°C for 30 min to 2 h. Constitutive colonies appear as bright yellow colonies while non-constitutive colonies are either white or a very pale yellow. All tests were performed with control *putA* (A$^+$C$^-$) and wild-type strains on the same Petri dish.

(f) *Tn*10 *mutagenesis*

Tn*10* mutagenesis was accomplished by allowing Tn*10* to insert randomly into the chromosome of *S. typhimurium* LT2 (Kleckner *et al.*, 1975,1977). Put$^-$ mutants were identified by replica printing to PSN minimal medium containing 10 μg tetracycline/ml. Insertions identified as being Put$^-$ (unable to grow on the PSN minimal medium but able to grow on succinate ammonia minimal medium) were purified once selectively and then once on rich media. Further analysis of these mutants is described in the text. The isolation of Tn*10* insertions near *put* has been described previously (Chumley *et al.*, 1979).

(g) *Tn*5 *mutagenesis*

Tn*5* mutagenesis was accomplished by growing bacteriophage P22 HT *int*$^-$ on TT1780, which contains a Tn*5* insertion in the *lacZ* gene on an F'*lac* plasmid. These phage were then used as donors to transduce to kanamycin resistance on rich plates. (The transposon Tn*5* encodes a protein conferring kanamycin resistance; Berg *et al.*, 1975.) Since LT2 shares no DNA sequence homology with the F'*lac* plasmid, any inherited kanamycin resistance must be due to the transposition of Tn*5*. This method of non-homologous transduction (NHT) gives a collection of insertions which include 0·5% auxotrophs. This frequency is similar to that observed for Tn*10* transposition from a specialized P22 phage (Kleckner *et al.*, 1977). The distribution of auxotroph types (data not given) and the map position of the Tn*5* insertions in the *put* genes (see Results) are consistent with a random collection of Tn*5* insertions. For the isolation of Tn*5* insertions in the *put* genes, the rich kanamycin-containing plates on which the NHT cross was performed were printed to *put* indicator

plates containing kanamycin. White colonies were purified and tested on both minimal glucose and PSN plates. The results are given below. For the isolation of Tn*5* insertions near the *put* genes, 3000 colonies from an NHT cross were pooled and grown in liquid culture. Phage P22 was grown on such pools and the lysate was used to transduce the deletion *putPA557* to Put$^+$ and kanamycin resistance. The clones that had simultaneously become Put$^+$ and kanamycin-resistant were purified and examined for the transductional linkages of Tn*5* to *put*. This method is strictly analogous to that described for the isolation of linked Tn*10* insertions (Chumley *et al.*, 1979). Several *put*-linked Tn*5* insertions were isolated by this method and are listed in Table 1.

(h) *Transductional mapping crosses*

In mapping the *put* region, crosses were performed using the P22 high-frequency transducing mutant HT105/1 *int*$^-$ (Schmieger, 1972; Scott *et al.*, 1975). Cells were infected with phage at a multiplicity of 1 plaque-forming unit per cell. Put$^+$ recombinants were scored after 2 to 3 days of incubation at 37°C on PSN media supplemented with 0·4% nutrient broth. New Put$^-$ point and insertion mutations were mapped onto the existing map of the *put* genes described by Ratzkin & Roth (1978). In our mapping, a mutation was considered to lie within the region of the map deleted in the recipient strain if no recombinants were seen in crosses that would have yielded more than 2000 recombinants with a wild-type (*put*$^+$) donor.

(i) *Placement of* put *mutations on the episome* $F'_{ts}601$

The $F'_{ts}601$ (F′*put*) episome and its isolation have been described previously (Chumley *et al.*, 1979). Mutations were placed on $F'_{ts}601$ by homogenotization using the following procedure. $F'_{ts}601$ was transferred from the strain TT1852 into the recipient *put*$^-$ mutants to be homogenotized. Selection was made for tetracycline resistance; $F'_{ts}601$ harbors the transposon Tn*10*. A single colony of the resulting diploid was used to inoculate 1 ml of nutrient broth. Following growth into stationary phase, cells were seeded for single colonies on *put* indicator plates containing tetracycline. Rare white colonies appeared among a background of red colonies at a frequency of 0·1 to 1%. Such white (Put$^-$) colonies were purified as potential homogenotes. The F′ episomes from these candidates were then transferred into the recipient strain TR2615 (*hisD6429 putPA544 recA-1*) selecting tetracycline resistance. The phenotype of the episomal *put* mutation was examined. For strains showing the correct phenotype, phage P22 lysates were prepared and used to demonstrate that the episomal *put*$^-$ mutations mapped in the same deletion interval as did the chromosomal mutation from which the original homogenotes were isolated. The *put* mutations *639*, *667*, *616*, *666*, *696*, *651*, *736*, *610*, *625* and *557* were all placed on $F'_{ts}601$ by this procedure. F plasmids with the *put*$^-$ mutations listed above were all maintained in the Rec$^-$ chromosomal background of TR2615 and assigned the strain designations given in Table 3.

Mutations *put-853*, *-835*, *-852*, *-841*, *-845*, *-837* and *-838* are all Tn*5* insertions. These mutations were transduced directly onto the plasmid $F'_{ts}601$ in the strain TT1869 (*putPA521 pyrD121*/$F'_{ts}601$) by phage P22-mediated transductional crosses. The mutation *putPA521* is a large deletion that is not transducible to *put*$^+$ by phage P22 grown on a strain with the wild-type *put*$^+$ genes (Ratzkin & Roth, 1978). Inheritance of any *put*-Tn*5* insertion mutation by homologous recombination in strain TT1869 must, therefore, take place on $F'_{ts}601$. Transductants inheriting kanamycin resistance were scored for their Put phenotype. The map position of all *put*-Tn*5* mutations placed on $F'_{ts}601$ was rechecked by P22-mediated transductional crosses to be certain that the F-plasmid carried the correct mutation.

The *put*$^+$ constitutive mutations *putA907* and *putA911* were both placed on $F'_{ts}601$ by the use of a Tn*5* insertion near *put* (*zcc-628*::Tn*5*). Phage P22 was grown on the strain TT2660 (*put-557 zcc-628*::Tn*5*) and used to transduce TR5434 (*putA907*) and TR5512 (*putA911*) to kanamycin resistance. Recombinants that retained their constitutive phenotype were

TABLE 1

Genotypes of all strains used

Strain designation	Genotype				
TR2615	*hisD6429*	*putPA544*	*recA-1*	*strA*^{R}	
TR5280	*proAB47*	*proP673*			
TR5431	*proAB47*	*proP673*	*putA903*	(A^+C^-)	(*putA741*?)†
TR5432	*proAB47*	*proP673*	*putA904*	(A^+C^-)	(*putA741*?)†
TR5433	*proAB47*	*proP673*	*putA905*	(A^+C^-)	(*putA655*?)†
TR5434	*proAB47*	*proP673*	*putA906*	(A^+C^-)	(*putA655*?)†
TR5508	*proP673*	*putA907* (A^+C^-)			(*putA742*?)†
TR5509	*proP673*	*putA908* (A^+C^-)			(*putA739*?)†
TR5510	*proP673*	*putA909* (A^+C^-)			(*putA739*?)†
TR5511	*proP673*	*putA910* (A^+C^-)			(*putA739*?)†
TR5512	*proP673*	*putA911* (A^+C^-) *putA748*			
TT1780	*pyrC7 leuD798 fol-101 supQ1238*/F′ *proB*$^+$ *lacZ*::Tn*5*				
TT1791	*put*$^+$	*zcc-5*::Tn*10*			
TT1797	*put*$^+$	*zcc-7*::Tn*10*			
TT1801	*putPA523 proAB47 proP673 zjd-27*::Tn*10*				
TT1852	*his06203 putPA544 recA1*/F′$_{ts}$601 *put*$^+$				
TT1869	*pyrD121 putPA521*/F′$_{ts}$601 *put*$^+$				
TT2272	*putPA524 zcc-7*::Tn*10*				
TT2653	*put*$^+$ *zcc-625*::Tn*5*				
TT2659	*put*$^+$ *zcc-628*::Tn*5*				
TT2660	*putPA557 zcc-628*::Tn*5*				
TT2772	*proAB47*	*proP673*	*putP837*::Tn*5*		
TT2773	*proAB47*	*proP673*	*putP838*::Tn*5*		
TT2777	*proAB47*	*proP673*	*putP849*::Tn*5*		
TT2778	*proAB47*	*proP673*	*putP852*::Tn*5*		
TT3232	*putC911 zcc-628*::Tn*5*				
TT3233	*pyrD121 putPA521*/F′$_{ts}$601 *putC911 zcc-628*::Tn*5*				
TT3234	*putC907 zcc-628*::Tn*5*				
TT3235	*pyrD121 putPA521*/F′$_{ts}$601 *putC907 zcc-628*::Tn*5*				
TT3237	*proAB47 proP673 putA901 zjd-22*::Tn*10*				
TT3238	*proAB47 proP673 putA902 zjd-27*::Tn*10*				
TT3239	*proAB47 proP673 putA903 zjd-27*::Tn*10*				
TT3240	*proAB47 proP673 putA906 zjd-27*::Tn*10*				
TT3241	*proAB47 proP673 putA907 zjd-27*::Tn*10*				
TT3242	*proAB47 proP673 putA908 zjd-27*::Tn*10*				
TT3243	*proAB47 proP673 putA911 zjd-27*::Tn*10*				
TT3244	*proAB47 proP673 putA915 zjd-27*::Tn*10*				
TT3245	*proAB47 proP673 putA920 zjd-27*::Tn*10*				
TT3251	*proAB47 proP673 putPA524 zcc-7*::Tn*10*				
TT3252	*proAB47 proP673 putPA524 putA844*::Tn*5*				
TT3401	*pyrB655*/F′$_{ts}$114 *lac*$^+$ *zzf-696*::Tn*5*				
TT3408	*pyrB655*/F′$_{ts}$114 *lac*$^+$ *zzf-703*::Tn*5*				

All strains are derivatives of *S. typhimurium* LT2 or LT7. The system for naming insertion mutations by their map position (e.g. *zcc-5*::Tn*10*) has been described (Chumley *et al.*, 1979). Strains with *put*$^-$ mutations on the episome F′ 601 are not listed in this Table but are given in Table 3. Strains TT1847, 1902, 1905, 1908, 1911, 1914, 2004, 2007, 2324, 2327, 2330 and 2336 are isogenic derivatives of TR2615. Strains TR2281, 2282, 2283, 2284, 2285, 2286 and 2287 are isogenic derivatives of strain TT1869.

† The presence of the original *putA*$^-$ mutation in these strains has not been tested. See Table 6.

purified and designated as TT3232 and TT3234. Phage P22 was grown on these strains and used to transduce the F′-carrying strain TT1869 to kanamycin resistance. Colonies inheriting kanamycin resistance were screened for the constitutive expression of proline oxidase. Recombinants identified as having co-inherited both Tn*5* and the constitutive *putA* mutations were demonstrated to co-transfer Tn*5* and the Put constitutive phenotype in conjugational crosses when the inheritance of tetracycline resistance was selected. Strains TT3233 and TT3235 were constructed using the above procedure.

(j) *Complementation tests among mutants defective for either* putA *or* putP *activity*

The complementation behavior of mutants defective for proline utilization was examined in strains made diploid for the *put* genes by the introduction of the episome $F'_{ts}601$. The placement of mutations on the episome $F'_{ts}601$ is described above. For episome transfer both donor and recipient strains were grown into mid-log growth phase in nutrient broth. Donor and recipient (50 μl of each) were mixed directly on selective media and streaked for single colonies from the donor/recipient mix. Selection was made for inheritance of the donor's tetracycline resistance on minimal media. The donor was counterselected due to its histidine or uracil requirement (some donor strains carried the *hisD6429* mutation while others carried the *pyrD121* mutation). Three well-separated individual exconjugant colonies were picked to the identical selective media. Following growth, these selective master plates were replica printed to PSN minimal media and *put* indicator plates. In all cases both Put^- and Put^+ exconjugants were included on the same master plate as controls. A diploid exconjugant was scored as Put^+ if all 3 colonies showed both luxuriant growth on PSN minimal media and a deep red color on *put* indicator plates. A diploid exconjugant was scored as Put^- if all 3 colonies showed poor or no growth on PSN-minimal media and were white on *put* indicator plates. In the few cases where all 3 colonies did not behave identically the complementation test was repeated with 6 exconjugant colonies where agreement was found. Since these tests are performed in a recombination-proficient background, we assume that the rare cases in which we observed a disagreement among the triplicate exconjugants can be accounted for by recombination between the chromosomal and episomal *put* regions. All growth is carried out at 30°C since the episome $F'_{ts}601$ is temperature-sensitive for replication (Chumley *et al.*, 1979).

(k) *Complementation tests among mutations constitutive for the expression of the* put *genes*

To examine the complementation behavior of constitutive mutations diploids were formed in a manner analogous to that used for Put^- mutants. Exconjugants were printed from their selective master plates to succinate/ammonia minimal plates. Replicas growing on the succinate plates were assayed for the constitutive expression of proline oxidase by the *O*-aminobenzaldehyde color method described earlier. Each diploid in which a chromosomal constitutive mutation was recessive to an allele of the *put* genes on $F'_{ts}601$ was single-colony-isolated on nutrient media. To allow $F'_{ts}601$ segregation, a single colony from the diploid was then grown in 1 ml of nutrient broth at 30°C. This liquid culture was diluted 10^{-5} and samples were grown at both 30°C and 42°C in nutrient broth. These cultures were plated for single colonies on nutrient broth and replica-printed on nutrient broth plus 50 μg kanamycin/ml. Colonies that had lost the episome $F'_{ts}601$ could be readily identified since they had become kanamycin-sensitive (the only copy of Tn*5* in these strains is present on the episome $F'_{ts}601$). Three colonies from the 42°C culture that have become kanamycin-sensitive and 3 colonies from the 30°C plates that remained kanamycin-resistant were picked to a master plate, which was then replica-printed to test for the constitutive expression of proline oxidase as described above. A complementation test is considered to have demonstrated the recessivity of a chromosomal constitutive mutation only when removal of the $F'_{ts}601$ episome restored the constitutive phenotype. No similar tests were performed for the

complementation crosses in which the constitutive mutation was harbored on the episome $F'_{ts}601$. We believe that the transfer of the mutant episome to deletion *putPA557* and to LT2 (*put*$^+$) provides sufficient controls in these cases.

(l) *Construction of strains used in the mapping of constitutive mutations*

The scheme we have employed to map constitutive mutations requires the construction of double mutants having in the *putA* gene both a Tn*5* insertion and the constitutive mutation to be mapped. Details of the mapping cross are given in Results and Fig. 2. Here we describe the construction of the donor double *putA* mutants used in the mapping crosses.

An initial step in the strain constructions is the formation of the strain TT3252 (*putPA524 putA844*::Tn*5 proP673 proAB47*). To accomplish this, the deletion *putPA524* was transduced to tetracycline resistance with phage P22 grown on TT1797 (*zcc-7*::Tn*10*). Recombinants were screened for the double-mutant phenotype of tetracycline resistance (Tn*10*) and azetidine resistance (*putP*$^-$). The linkage of Tn*10* to the deletion was demonstrated in one such double-mutant transductant TT2272 (*putPA524 zcc-7*::Tn*10*). Phage P22 grown on the strain TT2272 were then used to transduce the recipient TR5280 (*proAB47 proP573*; described by Menzel & Roth, 1980) to tetracycline resistance. Transductant clones were screened for recombinants that had co-inherited the deletion *putPA524*. One such transductant was purified and designated as TT3251 (*proAB47 proP673 putPA524 zcc-7*::Tn*10*). Phage P22 grown on TT2603 (*putA844*::Tn*5*) was used to transduce TT3251 to kanamycin resistance (Tn*5*). These transductants were then screened for recombinants that had lost their tetracycline resistance (Tn*10*) and retained the PutP$^-$ phenotype (*putPA524*). This recombinant is the desired strain TT3252.

The strain TT3252 has the property of being unable to have its proline requirement (due to the *proAB47* lesion) supplemented with 17 μM-proline. Growth on 17 μM-proline requires either a functional *proP*$^+$ or *putP*$^+$ permease gene in strains defective for the synthesis of their own proline (Menzel & Roth, 1980). We are able to exploit this property as it provides a basis for selecting a *putP*$^+$ allele without requiring proline as its sole nitrogen source. In the strain TT3252, selection of a *putP*$^+$ allele involves the replacement of all of the *putP* gene as well as the major portion of the *putA* gene since the PutP$^-$ phenotype is due to the deletion *putPA524*. If phage grown on *put* constitutive mutants are used to transduce TT3252 to low Pro$^+$ (growth on 17 μM-proline) while selection is also made for kanamycin resistance, then the double mutants with both the *put* constitutive mutation and the Tn*5* insertion mutation may be formed. When the donor constitutives are in a genetic background with both a functional *proAB*$^+$ gene and *proP*$^+$ gene, transduction to low Pro$^+$ may also give us *proAB*$^+$ and *proP*$^+$ transductants as well as the desired *putP*$^+$ transductants. To avoid this, the constitutives are first transduced into a background which is *proAB*$^-$ and *proP*$^-$. This is accomplished by transducing TT1801 (*proAB47 proP673 zjd-27*::Tn*10 putPA523*, described by Menzel & Roth, 1980) to growth on PSN media. The constitutives used in our mapping scheme were all first placed in this background and are listed in Table 1. These strains were used as donors to transduce strain TT3252 to growth on medium with 17 μM-proline producing the *put* constitutive-*putA844*::Tn*5* double mutants required by our mapping scheme.

(m) *Permease assays*

Cells for uptake studies were grown to a density of approximately 10^8 cells/ml in NCE minimal media supplemented with either 2% glucose or 0·4% succinate with and without 2 mM-proline. These cells were harvested by centrifugation and washed 3 times in buffer A (Tanaka *et al.*, 1967). For uptake assays, cells were resuspended to a concentration of between 100 and 400 μg cell protein/ml in buffer A supplemented with 11 mM-glucose. Cell protein was determined by the method of Lowry *et al.* (1951). Prior to an assay the cells (in buffer A plus glucose) were incubated for 5 min at 37°C. To initiate the assay, 400 μl of the cell suspension were added to the radioactive proline at a final concentration of 20 μM with a

specific activity of ^3H of 500 Ci/mol. Samples of 100 μl were filtered at 10, 30 and 50-s time intervals on cellulose-acetate, cellulose-nitrate filters (Millipore, 0·45 μm) with vacuum. Immediately after filtering, the cells were rinsed with 10 ml of a buffer containing 10 mM-Tris·HCl (pH 7·3), 0·15 M-NaCl and 0·5 mM-$MgCl_2$. Filters were dried and counted in a toluene-based scintillation fluid as previously described (Ratzkin & Roth, 1978). The amounts of label taken up between the 10 s and 30-s, and 30 s and 50-s time intervals were used to calculate the uptake rate. If the 2 values differed by more than 20% the results were disregarded and the assays repeated. All assays were done in triplicate with the reproducibility indicated in Results.

3. Results

(a) *Isolation of new* put *mutants*

To investigate further the genetics of proline utilization we isolated a number of new *put* mutants caused by Tn*5* insertion, Tn*10* insertion and hydroxylamine.

To distinguish the various phenotypes resulting from mutations mapping within the *putA* gene, we have adopted the following notation. All mutations mapping within the *putA* gene are given the *putA* designation followed by an allele number. In addition, the allele designation is followed by the letters A and C in parentheses. An A^+ designation in parenthesis indicates that the product of the mutant *putA* gene retains both enzymatic activities; A^- denotes a defect in enzyme activities. The C^+ designation indicates that the *putA* gene products' regulatory functions are normal; C^- indicates constitutive expression of both *putA* enzyme and *putP* permease activities in A^+C^- mutants and constitutive expression of only *putP* permease activity in A^-C^- mutants. Strains constitutive for permease (*putP*) are supersensitive to inhibition by AZ.

(i) *Tn*10 *mutants*

Among 20,000 Tn*10* insertions tested, ten put^- mutants were identified. All mutants were of the *putA* (A^-C^-) type; they failed to grow in response to Leu-Pro and proved to be supersensitive to AZ (AZ^{SS}). The failure to find $putP^-$::Tn*10* insertions may be due to the fact that $putP^-$ mutants are somewhat leaky and particularly difficult to score upon replica printing to PSN media. It is also possible that hotspots for Tn*10* insertions exist in *putA* (Kleckner *et al.*, 1979). Mapping data discussed below demonstrate at least four independent sites of Tn*10* insertion within the *putA* gene.

(ii) *Tn*5 *mutants*

Among 20,000 random Tn*5* insertion mutants, 20 put^- mutations were identified using the *put* indicator plates described in Materials and Methods. Among these, ten are $putP^-$ (AZ^R and grow on Leu-Pro), six are *putA* (A^-C^-) (AZ^{SS} and fail to grow on Leu-Pro) and four belong to a new mutant class *putP** (leaky Put^-, AZ^S and grow on Leu-Pro). The same 20,000 colonies were scored for the constitutive expression of proline oxidase as described in Materials and Methods. From the 20,000 random Tn*5* insertion mutants, no clones could be identified that were constitutive for the expression of proline oxidase.

(iii) *Localized mutagenesis of the* put *region by hydroxylamine*

Phage P22 was grown on TT1797 (*put*$^+$, *zcc-7*::Tn*10*; Tn*10* 80% linked to the *put* genes). The lysate was concentrated and mutagenized with hydroxylamine as described by Hong & Ames (1971). These mutagenized phage were used to transduce LT2 to Tet^R. Among 20,000 Tet^R transductants, 185 white colonies were identified using *put* indicator plates; 100 of these were picked, purified and characterized as follows: 34 *putA* (A^-C^-), 23 *putA* (A^-C^+), 31 *putP* and five *putP**. The *putA*, *putP* and *putP** mutants are defined by the criteria given above. The remaining seven mutants carried lesions unrelated to *put* expression. The same 20,000 colonies were scored for the constitutive expression of proline oxidase activity; 12 *putA* (A^+C^-) constitutive mutants were identified.

(iv) *The new* put *mutants*

A summary of the mutants isolated is given in Table 2. A striking feature of these results is that all *putA* insertion mutations are constitutive for the permease *putP* (no *putA* (A^-C^+) insertion mutations were found). Polar point mutations within the *putA* gene (frameshifts and amber types) also overproduce permease and thus become supersensitive to the proline analogue, azetidine carboxylic acid (AZ^{SS}). We will conclude that the *putA* gene product is itself involved in repression of the *putP* and *putA* genes.

All of the insertion mutations were defective in either *putP* or *putA*; none is defective for both *putP* and *putA*. Since the insertion elements used are strongly polar (Kleckner *et al.*, 1977), these results strengthen the conclusion of Ratzkin & Roth (1978) that the *putP* and *putA* genes are transcribed independently.

TABLE 2

Mutants isolated in three separate experiments

		Mutant types observed				
Mutagen	Isolation scheme	*putA* (A^-C^-)	*putA* (A^-C^+)	*putA* (A^+C^-)	*putP*	*putP**
Tn*10* insertion	Screen for proline non-utilization	19	0	Not tested	0†	0†
Tn*5* insertion	Screen on *put*-indicator medium Screen for constitutive oxidase	6	0	0	10	4
Hydroxylamine (local mutagenesis)	Screen on *put*-indicator medium Screen for constitutive oxidase	34	23	12	31	5‡

The procedural details of the mutagenesis are given in Materials and Methods and the accompanying text. A definition of the mutant types observed is given in the text.

† Permease mutants may be too leaky to detect by the methods used.

‡ These *P** mutants are different from the Tn*5* *P** mutant (see section (b), below).

The new class of *putP** mutants among the Tn*5* insertions presents an interesting problem. Phenotypically, *putP** mutants are simply leaky permease mutations. The hydroxylamine-induced mutants with the *putP** phenotype are easily interpreted because hydroxylamine causes base substitutions. The leaky Tn*5* insertion mutants are more difficult to explain since insertion of a Tn*5* element with a gene should not result in a slightly altered gene product. These mutants are still under study.

(b) *Map position of the new* put$^-$ *mutations*

(i) *Tn5 mutations*

All the Tn*5* mutations isolated were placed on the genetic map of the *put* region by transductional crosses with previously described deletion mutants by procedures given in Materials and Methods. The map presented in Figure 1 is a refinement of that published by Ratzkin & Roth (1978). All the *putA* (A^-C^-) mutations (*841*, *836*, *845*, *842*, *843*, *844*) map within the region of the map previously defined as the *putA* gene. The *putP* mutations (*849*, *852*, *835*, *847*, *851*, *854*, *834*, *845*, *848*, *853*) map within the region defining the *putP* gene. The *putP** insertion mutations (*837*, *839*, *840*, *838*) cluster in the region between the *putA* and *putP* genes.

(ii) *Tn10 mutations*

Only 14 of the 19 *putA*::Tn*10* mutations were mapped. Among these, the *putA* (A^-C^-) mutations *821*, *830*, *826*, *827*, *818*, *815* and *811* were mapped with sufficient resolution to place them on the genetic map in Figure 1. Mutations *829*, *824*, *823*,

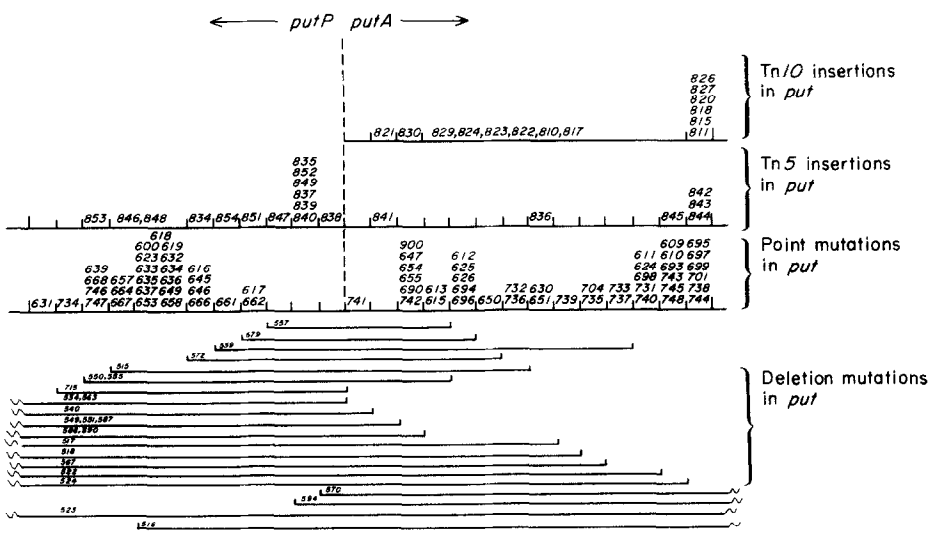

FIG. 1. Map of the *put* genes. The point mutations were ordered by Ratzkin & Roth (1978) using the indicated deletions. Tn*5* and Tn*10* insertion mutations isolated in this study were placed on the map by P22-mediated transductional crosses as described in Materials and Methods.

822, *810* and *817* were shown to lie within the previously defined *putA* gene (outside the deletion *putPA590* but within the region covered by the deletion *putPA524*). The Tn*10* insertion mutations map at a minimum of four sites within the *putA* gene.

(iii) *Hydroxylamine-induced mutations*

Of the hydroxylamine mutations, only the map position of *putP** mutations was investigated. We were able to show that all of these phenotypically leaky *putP* mutations mapped well within the *putP* gene (outside the deletion *putPA557* and under the deletion *putPA523*). Our aim was to see if they might map in the same region as the Tn*5* *putP** mutations. They did not and we assume that the hydroxylamine-induced mutations are merely leaky missense mutations in the *putP* gene. Further resolution of the map position was not pursued. The other hydroxylamine-induced *put* mutations were not mapped since several representatives of all the classes had been placed on the genetic map by Ratzkin & Roth (1978).

(iv) *The revised genetic map*

The genetic map in Figure 1 is an extension of the previously published map (Ratzkin & Roth, 1978) with only minor revisions. It is still clear that permease mutations cluster at one end of the region and define a *putP* gene. The *putA* gene is still defined by a group of *putA*$^-$ mutations at the other end of the region.

The *putP**::Tn*5* insertion mutations cluster at the *putA-putP* gene border in a region with several other Tn*5* insertions that are standard (non-leaky) *putP* mutations. The observation that 40% of the *put*::Tn*5* mutations map in this region suggests that it is either large or that it represents a hotspot for Tn*5* insertions.

The map positions of both Tn*10* and Tn*5* insertions in the *putA* gene clearly place the previously described *put* constitutive mutation, *putA900* (A^+C^-), between *putA* (A^-C^-) insertion mutations. Other *put* constitutive mutations also map within the *putA* gene (see below).

(c) *Complementation tests among* put$^-$ *mutations*

Complementation tests were done using a F′ *put* plasmid ($F'_{ts}601$) whose construction has been described (Chumley *et al.*, 1979). Various *put*$^-$ mutations have been placed on this plasmid either by transduction or homogenotization. These episomes were transferred into various *put*$^-$ mutations selecting tetracycline resistance ($F'_{ts}601$ carries a Tn*10* element). Following transfer, exconjugants were scored for their Put phenotype on both PSN minimal plates and *put* indicator plates. Procedural details are discussed in Materials and Methods.

Table 3 shows the results of a large number of such complementation tests. The data demonstrate that most mutations can be classified as being members of the "P" complementation group (are complemented by *putA*$^-$ mutations; are not complemented by *putP*$^-$ mutations) or the "A" complementation group (are complemented by *putP*$^-$ mutations; are not complemented by any *putA*). A few unusual *putA* mutations fail to be complemented by any *putA* or *putP* mutation

(P/A group). Below the complementation data in Table 3, the recipient mutations are classified according to their complementation group (A, P, or the unusual P/A group).

All mutations classified phenotypically as *putP* belong to the single *putP* complementation group. Among the donors and recipients tested are examples of frameshift, base-substitition and insertion mutations. We believe that a single complementation group in the *putP* gene indicates that the *putP* region codes for a single polypeptide product.

Mutations mapping in the *putA* region belong primarily to the A complementation group; a few belong to an unusual class that fails to complement either *putA* or *putP* mutations; these will be discussed in a later section. The *putA* mutations in the A complementation group are distributed across the map and the unusual complementors are interspersed among them. As with the *putP* mutations, the donor and recipient *putA* mutations tested in our complementation tests include frameshift, base-substitution and insertion mutations. The single *putA* complementation group indicates that the *putA* region codes for a single polypeptide. Further evidence for this conclusion will be presented elsewhere (Menzel & Roth, 1981).

(d) *Some* putA *mutants are constitutive for expression of the* putP *gene*

Two types of mutations result in the AZ^{SS} phenotype characteristic of mutants with constitutive levels of *putP* gene product. One type, represented by *putA900* (A^+C^-), is constitutive for the expression of both proline oxidase and proline permease activity (Ratzkin & Roth, 1978). Based on this observation, we believe that the AZ^{SS} mutants which lack oxidase/dehydrogenase activity would also prove to be constitutive for the expression of proline permease. The data given in Table 4 show a correlation between the AZ^{SS} phenotype and a reduced *induction ratio* of proline permease for those mutants tested. We are puzzled by the fact that absolute values for uptake rates correlate only weakly with drug sensitivity. (These results would be expected if AZ caused repression of the wild-type *putP* gene.) The *putA* (A^+C^-) and *putA* (A^-C^-) mutants chosen for the assays span the entire *putA* map. Based on data such as that in Table 4, we have equated the AZ^{SS} phenotype with the constitutive expression of proline permease for all *putA* AZ^{SS} mutants.

We have already noted that insertion mutations in *putA* are AZ^{SS}. Analysis of existing *putA* mutations suggests that other types of polar mutations are also AZ^{SS}. A list of other *putA* mutations tested for their level of AZ sensitivity is given in Table 5. Among nine *putA* mutations induced by ICR-191, eight are AZ^{SS}. (ICR-191 causes primarily frameshift mutations (Ames & Whitfield, 1966).) Of 21 *putA* mutations that were induced by base-substitution mutagens (hydroxylamine, nitrosoguanidine and diethyl sulfate) only 12 are AZ^{SS} while the remaining nine are AZ^{S}. Of the 12 AZ^{SS} base-substitution mutations, two (*putA736* and *putA738*) were shown to be amber. We conclude that null mutations in the *putA* gene result in a simultaneous loss of enzyme activity and repressor function. We believe that only missense mutations can lose enzymatic activity without losing repressor functions.

TABLE 3

Complementation tests among put *mutations*

Donors	TT *put*	734	853	639	668	746	747	557	664	667	618	621	623	653	622	649	653	616	646	606	661	662	852	849	741	841	654	655	690	742	613	615	612	625	626	694	696	736	836	651	739	704	735	733	731	746	610	693	748	844	695	697	699	736	744	LT2	▽557
	Recipients:	*putP*																							*putA*																																	
	2284/853	−	−	−		−	−	−	−	−				−		−	−		−	−	−	−	−	−	+	+	+		+	+				−	−	+	+	+	+	+	+		+	+	+	+		+	+	+	+		+	+	+	+	−
	1908/639	−		−	−	−		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−			+		+	+		+	+	−	−	−	−	+	+	+		+	+	+	+	+	+	+	−	+	+		+	+	+	+	+	+	−
putA	1914/667	−		−	−	−		−	−	−	−	−	−	−	−	−	−	−	−						+		+	+		+	+		−	−	−	+	+	+		+	+	+	+	+	+	+		+	+		+	+	+	+	+	+	−
mutations	1905/616	−		−	−	−		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−			+		+	+		+	+		−	−	−	+	+	+		+	+	+	+	+	+	+	−	+	+		+	+	+	+	+	+	−
	1911/666	−		−	−	−		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−			+		+	+	+	+	+	−	−	−	−	+	+	+		+	+	+	+	+	+	+	−	+	+		+	+	+	+	+	+	−
	2004/835	−		−	−	−		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−			+		+	+	+	+	+	−	−	−	−	+	+	+		+	+	+	+	+	+	+	−	+	+		+	+	+	+	+	+	−
	2283/852	−	−	−		−	−	−	−	−				−		−	−		−	−	−	−	−	−	+	+	+		+	+				−	−	+	+	+	+	+	+		+	+	+	+		+	+	+	+		+	+	+	+	−
	2286/841	+	+	+		+	+	+	+	+				+		+	+		+	+	+	+	+	+	−	−	−		−	−				−	−	−	−	−	−	−	−		−	−	−	−		−	−	−	−		−	−	−	+	−
putP	2330/696	+	+					+				+					+			+	+	+		+	−	−					−	−		−	−		−	−		−	−													−	−		
mutations	2324/736	+	+					+				+					+			+	+	+		+	−	−					−	−		−	−		−	−		−	−													−	−		
	1874/651	+		+	+	+		+	+	+	+	+	+					+	+						−			−			−	−	−	−		−		−		−	−			−		−	−	−	−		−	−	−	−	−	+	−
	2287/845	+	+	+		+	+	+	+	+				+		+	+		+	+	+	+	+	+	−	−	−		−	−				−	−	−	−	−	−	−	−		−	−	−	−		−	−	−	−		−	−	−	+	−
	2327/738	+	+					+				+					+			+	+	+		+	−	−					−	−		−	−		−	−		−	−													−	−		
Unusual																																																									
putA	1902/610	−		−	−	−		−	−	−	−	−	−	−	−	−	−	−	−	−	−	−			−			−	−	−	−	−	−	−	−	−	−	−		−	−	−	−	−	−	−	−	−	−		−	−	−	−	−	+	−
mutations	2336/625	−	−					−				−					−			−	−	−		−	−	−					−	−		−	−		−	−		−	−													−	−	+	−
Controls	1852/*put*$^+$	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	2007/▽557	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
	Group	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	A	A	A	A	A	A	A	P/$\bar{A}$	P/$\bar{A}$	P/$\bar{A}$	P/$\bar{A}$	A	A	A	A	A	A	A	A	A	A	A	P/$\bar{A}$	A	A	A	A	A	A	A	A	wt	P/$\bar{A}$

The Table summarizes a large number of complementation tests conducted with the *put* mutants listed. Recipient *put* mutants are listed across the top of the Table according to their map order. Donor strains are listed along the left-most column with both a strain designation and *put* allele number. The results of a complementation test are given at the intersection of a donor row and recipient column: a (+) indicates that the resulting diploid is phenotypically Put$^+$; a (−) indicates the diploid is phenotypically Put$^-$, and a blank indicates that diploid pair was not tested. At the bottom of the Table the recipient *put* mutants are assigned either to the P, A, or unusual P/A complementation group on the basis of their behavior in the test reported in this Table. For procedural details see Materials and Methods.

(e) *Analysis of* putA *revertants*

Since some *putA* mutations are *putP* constitutives (AZ^{SS}) and since the constitutive mutation *putA900* (A^+C^-) maps within the *putA* structural gene, we propose that the *putA* gene product acts as a repressor in the regulation of both the *putA* and *putP* genes. If this is the case, then some definite predictions can be made about the types of revertants expected for both *putA* (A^-C^+) and *putA* (A^-C^-) mutations. Alterations in the protein's structure that correct a defect in enzymatic activity may or may not alter the protein's regulatory function. We expect that most revertants would be to a wild-type protein with a minority class retaining a defect in regulatory activity. If a single protein is responsible for both the catalytic and regulatory activity of the *putA* gene product, then both *putA* (A^-C^+) and *putA* (A^-C^-) mutations might be expected to give rise to both $PutA^+C^+$ and $PutA^+C^-$ revertants. In Table 6 we show the fraction of revertants constitutive for the expression of proline oxidase activity among the spontaneous Put^+ revertants from a number of *putA* mutants. The results show that both *putA* (A^-C^+) and *putA* (A^-C^-) mutants can revert to *putA* (A^+C^-). However, for both *putA* (A^-C^+) and *putA* (A^-C^-) mutants the majority class of revertants are *putA* (A^+C^+). It seems reasonable that revertants that restore the "proper" conformation to a protein for enzyme activity will also, in the majority of cases, have a "proper" conformation for regulatory activity. Mutations that can give rise to constitutive revertants exist across the entire *putA* gene suggesting that all of the protein is important to its regulatory function. All revertants constitutive for proline oxidase proved to be constitutive for the permease (i.e. they are AZ^{SS}). We have tested the level of AZ sensitivity for eight non-constitutive (C^+) revertants of each of the following *putA* mutants: *putA655* (A^-C^+), *putA741* (A^-C^-), *putA736* (A^-C^-), *putA739* (A^-C^-), *putA735* (A^-C^-) and *putA738* (A^-C^-). All these revertants proved to be AZ^S. The reversion events of the type *putA* (A^-C^-) reverting to *putA* (A^+C^+) and *putA* (A^-C^+) reverting to *putA* (A^+C^-) demonstrate the simultaneous mutation of two phenotypes, enzyme activity and regulatory function, while selection was made for only a single phenotype, enzyme activity.

(f) *Map position of constitutive mutations*

Crosses were designed to map constitutive mutations with respect to various deletion mutations with endpoints in the *putA* gene. Donor strains carried the constitutive mutation and a Tn*5* insertion. Recipient strains were *putA* deletions. Selection was made for Put^+ recombinants and these were scored for the co-inheritance of the donor constitutive mutation (Fig. 2). If the constitutive mutation lies within the region of the map deleted in the recipient, all Put^+ recombinants must become constitutive (Fig. 2(a)). If the constitutive mutation lies outside the region of the map deleted in the recipient both constitutive and wild-type Put^+ recombinants can be recovered (Fig. 2(b)). The ratio of wild-type to constitutive recombinants will reflect the ratio of the distance between the deletion endpoint and the constitutive mutation to the distance between the constitutive mutation and the Tn*5* insertion. The observation of any wild-type Put^+ recombinants will indicate that the constitutive mutation lies outside the deletion interval in the recipient.

TABLE 4

Permease regulation in various putA *mutants*

Strain	*putA* allele	Phenotype	Proline uptake rate for cells grown in		Induction ratio
			+ Proline	− Proline	
LT2	*putA*$^+$	A^+C^+ (AZ^S)	6·0	3·0	2·0
TR2139	*putA900*	A^+C^- (AZ^{SS})	6·0	6·0	1·0
TR5512	*putA911*	A^+C^- (AZ^{SS})	5·9	7·4	0·8
putA655	*putA655*	A^-C^+ (AZ^S)	3·8	2·0	1·9
putA651	*putA651*	A^-C^+ (AZ^S)	7·1	3·4	2·1
TR5321	*putA744*	A^-C^+ (AZ^S)	5·7	2·2	2·6
TT2600	*putA841*::Tn*5*	A^-C^- (AZ^{SS})	3·2	3·7	0·9
TT2549	*putA836*::Tn*5*	A^-C^- (AZ^{SS})	4·3	5·4	0·8
TR5315	*putA738*	A^-C^- (AZ^{SS})	7·1	7·0	1·0
TT1801	*putPA532*	$A^-C^-P^-$ (AZ^R)	<0·5	<0·05	—

The transport of proline into the cells of the mutants listed was measured as described in Materials and Methods. The values reported are in nmol/min per mg of cell protein. The results are reproducible at the level of ±10%. Uptake was measured for cells grown in NCE media supplemented with 2% glucose with (+proline) or without (−proline) 2 mM-proline. The induction ratio is the ratio of the level found in cells grown with proline divided by the level found in cells grown without proline.

TABLE 5

Azetidine carboxylic acid sensitivity of various putA *mutants*

put mutation	Mutagen used to induce mutation	AZ sensitivity
Controls		
put$^+$ (A^+C^+)	—	AZ^S
putA900 (A^+C^-)	—	AZ^{SS}
putP662	—	AZ^R
putA mutation		
696; (A^-C^+)	ICR	AZ^S
613, *614*, *611*, *624*, *598*, *697*, *699*, *701*; (A^-C^-)	ICR	AZ^{SS}
654, *655*, *630*, *651*, *737*, *609*, *743*, *745*, *744*; (A^-C^+)	HA or NG or DES	AZ^S
741, *742*, *650*, *732*, *739*, *735*, *733*, *731*, *740*, *748*, *736**, *738**; (A^-C^-)	DES or HA	AZ^{SS}

The level of AZ sensitivity was determined for various *putA* mutants as described in Materials and Methods. The assignment of AZ^S or AZ^{SS} was made on the basis of comparison with control strains. AZ^S designates normal or wild-type sensitivity to AZ and AZ^{SS} designates supersensitivity to AZ. Mutants with an asterisk are amber mutations. ICR, ICR-191; NG, nitrosoguanidine; DES, diethyl sulfate.

TABLE 6

Reversion of putA *mutations*

putA mutation	Phenotype	Fraction of Put$^+$ revertants constitutive for the expression of proline oxidase	Constitutive revertants assigned *put* allele number
putA654	(A^-C^+)	0/48	
putA655	(A^-C^+)	7/48	*putA903*, *putA904*
putA741	(A^-C^-)	13/48	*putA905*, *putA906*
putA742	(A^-C^-)	1/24	*putA907*
putA696	(A^-C^+)	0/24	
putA736	(A^-C^-)	0/48	
putA630	(A^-C^+)	0/24	
putA651	(A^-C^+)	0/24	
putA739	(A^-C^-)	3/24	*putA908*, *putA909*, *putA910*
putA735	(A^-C^-)	0/24	
putA731	(A^-C^-)	0/24	
putA740	(A^-C^-)	0/24	
putA748	(A^-C^-)	1/24	*putA911*
putA738	(A^-C^-)	0/48	

The *putA* mutants listed were reverted to A^+ by plating 10^8 mid-log cells on PSN media. No mutagens were used. Revertant, *putA*$^+$, colonies were picked, purified and then scored for the constitutive expression of proline oxidase as described in Materials and Methods. The fraction of constitutive revertants is given in the Table along with the allele number assigned to the constitutive revertants, which were saved and used in subsequent experiments. The assigned allele numbers refer to the second correcting lesions; it should be noted that these strains may still carry the parental *putA* mutation.

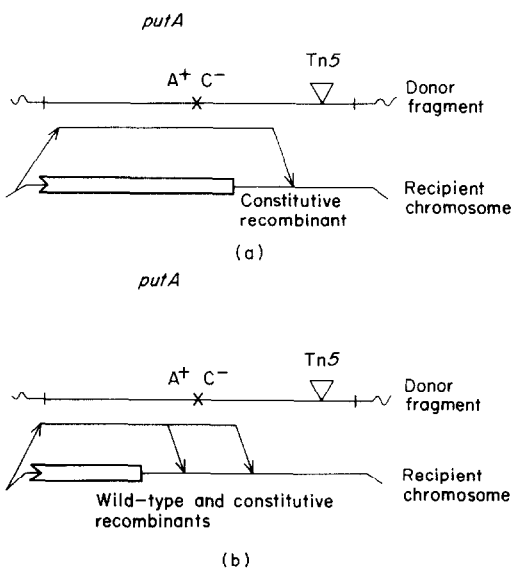

FIG. 2. Constitutive mapping scheme. The Figure illustrates the scheme employed to map constitutive mutations. (a) The expectations for recombinants when the recipient deletion includes the region in which the constitutive mutation is located. (b) The expectations for recombinants when the constitutive mutation lies outside the region deleted in the recipient. See Discussion in text.

TABLE 7

Mapping data for putA *constitutive mutations*

Recipient deletion	Constitutive *putA* mutation used as donor (fraction of Put$^+$ recombinants showing non-constitutive phenotype)	
	911†	*913*‡
590	8/50	35/50
557	7/50	36/50
679	7/50	35/50
572	2/50	20/50
515	0/50	20/50
518		18/50
567		18/50
522		7/50
524	0/50	0/50
523	no transductants observed	

The Table lists the results of crosses performed with the indicated donor mutations and recipient deletions. Details of the experiments are given in the accompanying text. Refer to the genetic map (Fig. 1) for position of the deletions and conclusions from this data.

† This mutation was isolated as a revertant of a *putA* mutation; see Table 6.

‡ This mutation was isolated by localized mutagenesis; see Table 2.

NOTE: Some deletions have endpoints to the left of *putPA590* (Fig. 1) and are unusable in this mapping scheme since they contain the mutation *putA900* (A^+C^-).

A number of double-mutant strains were constructed and crossed with several deletions. Put$^+$ recombinants were purified selectively and scored for the constitutive expression of proline oxidase. Eight of the constitutive mutations failed to give wild-type Put$^+$ recombinants with *putPA590* and presumably lie at the left end of the *putA* gene. The results of crosses are given in Table 7 for two other constitutive mutations. We have been able to demonstrate clearly that *putA911* (A^+C^-) and *putA913* (A^+C^-) map within the *putA* gene. Including the original constitutive mutation, *putA900* (A^+C^-), we have three different mutations that are constitutive for both oxidase/dehydrogenase and permease, and clearly map within the *putA* gene. These three mutations span the genetic map of the *putA* gene demonstrating that all of the *putA* gene product is important to the proper function of the *putA* gene product in regulation. The mutation *putA911* (A^+C^-) is particularly interesting since it is a revertant of the *putA*$^-$ mutation *putA748* (A^-C^-). The deletion interval in which the constitutive phenotype maps is far from the interval in which the original mutation maps. We have been able to

demonstrate that the original isolate of *putA911* (A^+C^-) still has the *putA748* (A^-C^-) mutation (Menzel, unpublished observation). (Note: the *putA*$^-$ mutation maps to the right of the constitutive-suppressor mutation and hence does not interfere with the mapping scheme.) The constitutive mutation *putA911* (A^+C^-) thus not only confers a constitutive phenotype on the *put* genes when present with *putA748* but is also able to suppress the *putA748* mutation intragenically.

(g) *Complementation behavior of constitutive mutations*

Donor F′*put* episomes harboring various *put* alleles were transferred into the *put* constitutive mutants listed in Table 8. All the constitutive mutations tested are recessive to a wild-type copy of the *put* region. An episome carrying a small internal deletion removing both *putA* and *putP* activities (*putPA557*) fails to correct the regulatory defect of the constitutive mutants. To demonstrate that the diploids constructed with the *put*$^+$ episome still carry the chromosomal constitutive mutation, the strains were retested following segregation of the F′ plasmid (described in Materials and Methods). All complementing diploids retain a chromosomal copy of the constitutive mutation. The results demonstrate that a regulatory element encoded in the plasmid-borne *put*$^+$ gene cluster is able to act *in trans* to repress the chromosomal *put* genes. Such *trans* correction is consistent with a repressor type control system (Jacob & Monod, 1961; Epstein & Beckwith, 1968).

To determine more precisely which element of the *put* gene cluster was responsible for the repressor activity, plasmids carrying either the mutation *putP853*::Tn*5* or *putA845*::Tn*5* were transferred into a few representative *put* constitutive mutants. The results, shown in Table 8, demonstrate that a good copy of the *putA* gene is necessary for regulatory activity: the *putP* mutant but not the *putA* mutant is able to correct the regulatory defect of the chromosomal constitutive mutations. Again, by plasmid segregation we were able to demonstrate that the constitutive mutations were still present in the chromosome of the diploids constructed with the *putP* mutant F′ plasmid.

Two types of *putA*$^-$ mutations exist: *putA* (A^-C^+) and *putA* (A^-C^-). We have argued that the *putA* (A^-C^-) mutants do not have the repressor activity while *putA* (A^-C^+) mutants retain their repressor activity. If this is true, one would predict that the *putA* (A^-C^+) mutants could provide repressor activity *in trans* in a complementation test while the *putA* (A^-C^-) mutants would not. We have tested this prediction by constructing F′-plasmids carrying constitutive mutations and transferring them into a variety of *putA*$^-$ mutants. The results, given in Table 9, show that *putA* (A^-C^+) mutants but not *putA* (A^-C^-) mutants are able to provide functions that repress the constitutive synthesis of proline oxidase for both *putA907* and *putA911* (A^+C^-) mutants.

(h) putP::*Tn5 mutants*

An interesting problem is presented by the "leaky" Tn*5* insertion mutations that lie in the *putA*-proximal side of the *putP* gene. How can insertion mutations result in only a partial loss of permease expression? To investigate further the nature of

TABLE 8

Complementation behavior of chromosomal put *constitutive mutants*

Constitutive mutation *putA* (all (A^+C^-)) used as recipient	Isolation scheme of indicated *putA* recipient	Uninduced *putA* enzyme level found in the diploid formed with the indicated episomal *put* allele			
		put$^+$	*putPA557*	*putP853*::Tn*5*	*putA845*::Tn*5*
putA907	A^+C^- revertant of *putA742*	+	−	+	−
putA908	A^+C^- revertant of *putA739*	+	−	+	−
putA909	A^+C^- revertant of *putA739*	+	−	+	−
putA903	A^+C^- revertant of *putA655*	+	−		
putA906	A^+C^- revertant of *putA741*	+	−		
putA910	A^+C^- revertant of *putA739*	+	−		
putA911	A^+C^- revertant of *putA748*	+	−		
putA900	NG	+	−	+	−
putA901	HA	+	−	+	−
putA902	HA	+	−	+	−
putA920	HA	+	−	+	−
putA913	HA	+	−		
putA914	HA	+	−		
putA915	HA	+	−		
putA916	HA	+	−		
putA917	HA	+	−		
putA918	HA	+	−		
putA923	HA	+	−		

The recipient *put* constitutives were made diploid with the indicated donor episome and the phenotype of the resulting diploid was determined. Procedural details are given in Materials and Methods and the accompanying text. NG, nitrosoguanidine; HA, hydroxylamine; +, repressed level; −, constitutive level.

TABLE 9

Complementation behavior of episomal put *constitutive mutants*

Recipient *putA* mutant	Phenotype of haploid recipient	Phenotype of diploid formed with indicated F′ plasmid mutation	
		F′ *putA907* (A^+C^-)	F′ *putA911* (A^+C^-)
putA654	(A^-C^+)	+	+
putA655	(A^-C^+)	+	+
putA651	(A^-C^+)	+	+
putA744	(A^-C^+)	+	+
putA841::Tn*5*	(A^-C^-)	−	−
putA742	(A^-C^-)	−	−
putA736	(A^-C^-)	−	−
putA739	(A^-C^-)	−	−
putA738	(A^-C^-)	−	−
putP853::Tn*5*	($A^+C^+P^-$)	+	+
putPA557	($A^-C^-P^-$)	−	−
put$^+$	($A^+C^+P^+$)	+	+

The recipient *putA* mutants were made diploid with the indicated donor episomes harboring a constitutive mutation and the phenotype of the resulting diploid was scored. Procedural details are given in Materials and Methods and the accompanying text. +, Repressed expression; and −, constitutive expression of the *putA* gene product.

these *putP*::Tn*5* insertions we have examined the levels of proline uptake in a number of these mutants. Rates of proline uptake were measured under the conditions of catabolite repression (glucose-grown cells) and non-catabolite repression (succinate-grown cells). The proline uptake rates of a strain with the $putP^+$ allele (shown in Table 10) indicate that the wild-type *putP* permease undergoes a 5·4-fold increase on the succinate media demonstrating that the $putP^+$ gene is under catabolite repression control. Both *putP** and $putP^-$::Tn*5* insertions fail to show any increase in permease activity when grown on succinate media.

TABLE 10

Proline uptake in putP *and* putP* *Tn5 insertions*

Strain	*putP* allele	Proline transport rate for cells grown in media: With glucose	With succinate	AZ sensitivity
TT2772	*putP*837*::Tn*5*	3·8	3·7	AZ^S
TT2773	*putP*838*::Tn*5*	8·5	5·8	AZ^S
TT2777	*putP849*::Tn*5*	2·6	2·1	AZ^R
TT2778	*putP852*::Tn*5*	1·7	1·6	AZ^R
TR5280	$putP^+$	7·0	38·0	AZ^S
TT1801	*putPA523*	<0·05	<0·10	AZ^R

The transport of proline into cells of the mutants listed was measured as described in Materials and Methods. The values reported are in nmol/min per mg of cell protein. The results are reproducible at the level of ±10%. Uptake was measured for cells grown in NCE media supplemented with 2 mM-proline and with either 2% glucose or 0·4% sodium succinate.

Regardless of the nature of the promoter in these mutants, it is clear that normal regulation is lost. The *putA*-proximal *putP*::Tn*5* insertions must lie in a region near the normal *putP* promoter and either supply a new promoter, insensitive to catabolite repression, or modify the regulatory signals seen by the normal *putP* promoter. The relative uptake rate of the *putP**::Tn*5* mutants on succinate media is only slightly higher than that of the $putP^-$::Tn*5* insertions. It is surprising that such a small difference can result in the *putP**::Tn*5* mutants being AZ^S (average level 4·7 nmol proline taken up/min per mg cell protein) and $putP^-$::Tn*5* mutants being AZ^R (average level 1·9 nmol/min per mg). The permease levels in both sorts of Tn*5* insertion mutants are clearly above that seen in a permease deletion strain.

The complementation behavior of *putP849*::Tn*5* and *putP852*::Tn*5* mutants is given in Table 3. Both these mutants behave in a manner identical to other *putP* mutants and belong to the P complementation group. The complementation behavior of *putP** mutants was also investigated. *putP** mutants are complemented by wild-type and $putA^-$ mutant plasmids but are not complemented by $putP^-$ mutant plasmids (data not shown). They are unusual in that they do complement themselves. Presumably the *putP** mutants have permease levels that border on being Put^+ and the diploid gene dosage is sufficient to provide enough permease to be scored as Put^+.

(i) *Unusual* putA *mutants*

From the complementation data in Table 3, it is clear that five *putA* mutants are unusual in their complementation behavior. Unlike other *putA* mutants, these mutants fail to complement not only other *putA* mutants but also *putP* mutants. However, these mutants are recessive *in trans* to a wild-type copy of the entire *put* region (*putA*$^+$ and *putP*$^+$).

A trivial explanation of this behavior would be the presence of two mutations in the strains having the unusual pleiotropic phenotype (*612*, *615*, *625*, *626* and *610*). One mutation, we would postulate, affects expression of the *putA* gene and a second affects the expression of *putP*. The deletion mapping that places the unusual *putA* mutations on the genetic map in Figure 1 rules out the possibility that these *putA* mutants have a second mutation in the *putP* gene. All these mutants are able to recombine with deletions that eliminate all of the *putP* gene.

We have carefully re-examined the phenotypes of these mutants with respect to the utilization of the dipeptide Leu-Pro and resistance to the proline analogue AZ. All are unable to utilize Leu-Pro. Mutants *610*, *612* and *615* are resistant to AZ. Mutants *625* and *626* show a wild-type level of AZ sensitivity. Permease assays verify that mutants *610*, *612* and *615* do not have *putP* activity (data not given). Permease assays also show that mutants *625* and *626* have *putP* activity. However, the levels are slightly reduced and do not fully derepress relative to a wild-type copy of the *put* genes (data not given).

We have examined the ability of these mutants to revert to Put$^+$. Mutants *612*, *615*, *625* and *626* fail to give spontaneous Put$^+$ revertants at a detection level of one in 10^{10}. The mutant *610* gives spontaneous Put$^+$, revertants at a frequency of 5 to 50 in 10^{10}. The mutant *610* is stimulated to revert by ICR-191 but not nitrosoguanidine. Mutants *612*, *615*, *625* and *626* fail to be stimulated to revert by either mutagen. The ability of these mutants to revert for *putA* activity alone was tested by their ability to revert to growth on plates containing 0·2% Leu-Pro as sole nitrogen source. Again only *putA610* was able to revert (to Leu-Pro growth). The other unusual *putA* mutants failed to revert spontaneously or to be induced to revert by mutagens. Eight spontaneous *putA610 leu-pro*$^+$ revertants were purified and shown to have regained both *putA* and *putP* functions; all eight revertants selected for Leu-Pro growth became Put$^+$ and AZ-sensitive. The mutation *putA610* is clearly a single mutation mapping in the *putA* gene, which inactivates both *putA* and *putP* activities. Failure of the other unusual *putA* mutations to revert suggests, but does not demonstrate, that they are small deletions. The reversion behavior of *putA610* suggests that it is a frameshift. We suspect that these mutations are strong *cis*-acting super-repressor mutations in the *putA* gene. (This is possible for the *putA610* frameshift since it maps at the end of the *putA* gene.)

(j) *Unusual* putP *mutants*

Mutants in the *putP* gene that are defective for both *putP* and *putA* activity were previously described. These mutants were reported to be resistant to AZ and unable to have their nitrogen requirement supplemented by Leu-Pro. We have

further analyzed these mutants and found that all grow very poorly on ammonia/succinate media. The phenotype of these strains is due to a *putP* mutation and a second unlinked mutation causing poor growth on succinate-containing media. Therefore, no single mutations are known in the *putP* gene that cause a defect in both *putP* and *putA* functions; *putA* mutations lacking both functions were described above.

4. Discussion

The major conclusion of this paper is that the *putA* gene product serves not only as a bi-functional proline-oxidizing enzyme but also as a repressor of both the *putA* and *putP* genes. The regulatory role of the *putA* gene is based on the following observations.

(1) Nonsense and insertion mutations in the *putA* gene (A^-C^-) have lost both enzymatic activities and are constitutive for expression of the *putP* permease. Loss of *putA* product results in loss of repression of *putP*.

(2) Certain point mutations within *putA* (A^+C^-) retain both enzymatic activities and are constitutive for expression of those activities and the *putP* permease. Alteration of *putA* can result in loss of repression ability without loss of enzymatic function.

(3) Certain point mutations within *putA* (A^-C^+) have lost both enzymatic activities but retain the ability to regulate *putP* permease. Apparently constitutive expression of *putP* is not a metabolic response to loss of the *putA* activities; some other qualitative alteration of the *putA* protein results in constitutivity.

(4) In complementation tests, A^-C^+ alleles of *putA* can supply repression control *in trans* to an A^+C^- allele.

We have purified the *putA* gene product and made a number of observations on its properties that will be reported in detail elsewhere (Menzel & Roth, 1981). The *putA* gene is a bifunctional membrane-bound 132×10^3 molecular weight polypeptide, which is present as a dimer of identical subunits following purification. The membrane association of the protein is based on the following observations.

(1) Upon the breakage of cells both proline oxidase and pyrroline-5-carboxylic acid dehydrogenase activities are associated with a particulate fraction. This has been reported previously (Frank & Ranhand, 1964).

(2) In order to purify the enzyme we must first solubilize it from the particulate fraction by the use of a non-ionic detergent. The solubilization destroys the oxygen-dependent oxidase activity. The solubilized enzyme requires the addition of an artificial electron acceptor for proline oxidase activity.

A speculative model provides a rationale for a membrane-bound protein that represses its own synthesis. The *putA* product is dependent on the presence of a membrane-bound electron transport chain, which uses oxygen as its terminal electron acceptor. We postulate that there are sites on the membrane with which the *putA* gene product must interact to become enzymatically functional. Once these functional sites have been "titrated", excess *putA* gene product (not membrane-bound) may act as a repressor of the *put* genes. Proline may interact with the *putA* protein to promote its insertion into the membrane. According to

this model, the *putA* (A^+C^-) mutant class may include mutant proteins that can insert into the membrane in the absence of proline, thus removing repressor and causing high constitutive levels of enzyme synthesis.

The concept of "titration of functional sites" finds a precedent in the case of the gene *32* product of phage T4 (Russel *et al.*, 1976; Lemaire *et al.*, 1978). The T4 gene *32* product is a single-stranded DNA binding protein, which is autogenously controlled. In the model of the regulation of T4 gene *32* product, Gold and co-workers (Russel *et al.*, 1976; Lemaire *et al.*, 1978) propose that gene *32* product is synthesized until all the single-stranded DNA binding sites are titrated. At this point the excess gene *32* product binds to its own message turning off the synthesis of more gene *32* product at the translational level. Whether the *putA* gene products' regulation proceeds by an analogous titration of functional sites will require further analysis. Autogenous control by titration of functional sites may be a general strategy used by many systems. Other examples have been reported by Dean & Nomura (1980), Nomura *et al.* (1981) and King *et al.* (1978).

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