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Cluster of Genes Controlling Proline Degradation in *Salmonella typhimurium*

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A cluster of genes essential for degradation of proline to glutamate (*put*) is located between the *pyrC* and *pyrD* loci at min 22 of the *Salmonella* chromosome. A series of 25 deletion mutants of this region have been isolated and used to construct a fine-structure map of the *put* genes. The map includes mutations affecting the proline degradative activities, proline oxidase and pyrroline-5-carboxylic dehydrogenase. Also included are mutations affecting the major proline permease and a regulatory mutation that affects both enzyme and permease production. The two enzymatic activities appear to be encoded by a single gene (*putA*). The regulatory mutation maps between the *putA* gene and the proline permease gene (*putP*).

Salmonella typhimurium and *Escherichia coli* can utilize proline as sole carbon or nitrogen source. In both species, the genes required for utilization of proline (*put*) map between the *pyrC* and *pyrD* genes at min 22 of the revised chromosome maps (5). Encoded in this region are structural genes for the major proline permease and the two degradative enzymatic activities, proline oxidase and pyrroline-5-carboxylic acid (PCA) dehydrogenase (16). To understand the organization and regulation of these genes, a fine-structure map of the region has been constructed. This map was constructed through use of a series of 25 deletion mutations and 70 point mutations affecting the region.

The necessary deletion mutants were selected as mutants resistant to one or more proline analogs. The analogs, azetidine-5-carboxylic acid (AC), 3,4-dehydroproline (DP), and baikiain (4,5-dehydro-L-pipecolic acid) seem to enter the cell via the proline permease, since a high percentage of mutants resistant to these analogs are defective in proline uptake. The analog baikiain inhibits only cells which are growing with constitutively high levels of proline oxidase (8). Presumably proline oxidase is required to metabolize baikiain to a toxic form. Baikiain-resistant mutants are defective in either proline permease or proline oxidase or both. Spontaneous mutants resistant to any of the above analogs can be selected easily; greater than 10% of the mutants isolated carry deletions.

Using deletions obtained through use of these analogs and a series of point mutations defective in proline utilization, a map of the *put* gene cluster has been made. This map permits several observations to be made concerning regulation and gene-enzyme relationships of the *put* genes.

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MATERIALS AND METHODS

Strains. All strains used in this paper are derivations of *S. typhimurium* strain LT2 except for TR1995, which is derived from strain LT7. Strains obtained from other sources and multiply marked strains are listed in Table 1. The *put* mutants isolated in the course of this work are listed in Table 3. This table will be described more fully later.

Media. Nutrient broth (NB; 0.8%) (Difco) with added NaCl (0.5%) was used as maximal medium. Minimal salts medium was the E medium of Vogel and Bonner (24). Minimal succinate-ammonia medium in which succinate is sole carbon source was E medium as modified by Berkowitz et al. (3); succinate was used at a concentration of 0.2%. For minimal proline medium in which proline is sole nitrogen source (succinate-proline), the medium of Davis and Mingioli (6) was used without glucose, citrate, or ammonium sulfate; succinate (0.2%) was added as sole carbon source, and proline (0.2%) was present as sole nitrogen source. Reversion tests and transduction tests in which the Put$^+$ phenotype was selected were performed on succinate-proline medium supplemented with 0.03% NB (wt/vol), 0.2 mM lysine, and 0.2 mM methionine. In solid media, agar was used at 1.5%.

Mutagenesis. (i) Diethyl sulfate. Diethyl sulfate mutagenesis was performed as described previously (18). Following mutagenesis, cells were usually grown for 10 generations to permit expression of mutant phenotypes. An exception to this was in isolation of *put* mutants by method 5 (see below); in this case, cells were grown for one to two generations following mutagen treatment. The mutants isolated by this method were considered as independent even though

TABLE 1. *Strains of S. typhimurium*

Strain	Genotype	Source
TR1995	*proAB47 putP639 proP673*	Ratzkin et al. (16)
TR2139	*putC900*	W. J. Brill (7)
TR2140	*putA609*	W. J. Brill (7)
TR2230	*hisO1242 hisC3734 hut⁺ galE542*	This paper
TR2613	*pyrC7 putC900*	This paper
TR3515	*pyrC1502*(Ts) *purC7 upp-102 put-521*	This paper
TR3684	*galE542 hut⁺*	B. A. D. Stocker

derived from a single culture.

(ii) NG. A 0.1-ml sample of an overnight culture in NB medium was spread on minimal glucose medium containing 0.4 mM AC. A small crystal of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) was placed in the center of the plate. After 2 days of incubation at 37°C, resistant colonies appeared in a ring surrounding the NG. Such colonies were considered to be independent mutants and were picked and purified for further testing.

(iii) ICR compounds. Both ICR191 and ICR364-OH were obtained from Hugh Creech of the Institute for Cancer Research, Fox Chase, Philadelphia, Pa. A 0.1-ml portion of an overnight culture in NB was used to inoculate 0.9 ml of minimal glucose medium containing 10 μg of the ICR compound. This culture was incubated in the dark at 37°C and allowed to grow to stationary phase. This mutagenized culture was then subjected to mutant selection procedures.

The frameshift mutagen hycanthone was used for some reversion tests. It has been described and was obtained from P. E. Hartman (11).

Mutant isolation. The following methods were used in isolation of *put* mutants. The number of the various methods will be used in Table 3 to describe how each *put* mutant was obtained.

Method 1: AC resistance. An overnight culture (0.1 ml) was spread on minimal salts medium. A few crystals of AC were added to the center of the plate. After 2 to 3 days of incubation, AC-resistant colonies appeared in the zone of growth inhibition surrounding the AC. These colonies were picked and characterized. Most proved to be *put* mutants.

Method 2: DP resistance. This method is identical to method 1 except that DP is used in place of AC.

Method 3: baikiain resistance. This method is identical to method 1 except that baikiain is used in place of AC. Since only strains constitutive for *put* expression are sensitive to baikiain, the regulatory mutant *putC900* is used as the parental strain for these selections.

Method 4: penicillin selection. This is a standard isolation procedure for obtaining mutants unable to utilize proline as nitrogen source. Penicillin selection was performed as described previously (18). After penicillin treatment, cells were plated for single colonies on minimal proline medium containing 0.4% NB (vol/vol). On this medium *put⁺* cells form large colonies; *put* mutants form only tiny colonies before exhausting the NB. The tiny colonies are picked and characterized.

Method 5: automated mutant selection. Several mutants were isolated by using a device for automated mutant identification developed by Donald Glaser and co-workers at the Facility for Automated Experiments in Cell Biology, Berkeley, Calif. These experiments were performed by Carol Greiner and John Creech. *put* mutants were selected from a mutagenized culture by direct observation of a large number of colonies. The mutagenized culture was plated for single colonies on medium containing proline as sole nitrogen source. This array was scanned automatically following several days of growth. The plates were then sprayed with a nitrogen source, $(NH_4)_2SO_4$, allowed to grow, and then scanned again. Colonies were automatically noted if they failed to grow on proline as sole nitrogen source but grew after addition of $(NH_4)_2SO_4$. The resulting mutants were screened for defects in proline utilization. Several *put⁻* mutants were obtained in this way (see Table 3).

Method 6: localized mutagenesis. This method is basically that of Hong and Ames (12), except that the high-transducing P22 mutant *HT105/1 int* was used (19). This phage carries both a mutation causing a high-frequency transducing ability (*HT105/1*) and an *int* mutation (isolated by Gary Roberts) which prevents integration and stable lysogeny. A phage lysate is prepared on a wild-type host and then mutagenized with hydroxylamine for 25 h (approximately 1% survival) (22). This phage lysate is used to transduce strain TR1995 (*proAB47 putP639 proP673*), selecting for ability to utilize a low (0.2 mM) concentration of exogenous proline to supply the proline requirement. The rationale for the selection is described in the text.

Method 7: auxotrophs requiring a high concentration of proline. Starting with deletion mutant *proAB47*, mutants were selected which fail to grow on 0.2 mM proline but can grow on 8 mM proline. The resulting mutants are defective in proline uptake.

Transductional crosses. In mapping the *put* region, crosses were performed using the P22 mutant *HT105/1 int* (19). Cells were infected with phage at a multiplicity of 1 plaque-forming unit/cell, and Put⁺ recombinants were scored after 2 to 3 days of incubation at 37°C. Mapping crosses were performed on succinate-proline medium containing 0.4% NB and both lysine and methionine (0.2 mM). No abortive transductants were seen; this may reflect the high level of *put* expression required for growth on proline as sole nitrogen source.

Reversion tests. A 0.1-ml portion of an overnight NB culture was spread on succinate-proline medium, and the mutagen to be tested was added to the center of the plate. In the case of NG, a small crystal was used; for ICR191 or ICR364OH, 0.01 ml of a solution (1 mg/ml) was used. In tests of the ICR compounds, plates were incubated in the dark. Reversion tests were first scored after 3 to 4 days; if no revertants appeared, incubation was continued for 1 week prior to scoring.

Enzyme assays. The proline permease and oxidase activities were assayed as described in the accompanying paper (16). The PCA dehydrogenase activity

was assayed as follows. Cells were harvested from a 25-ml culture and washed once with 5 ml of 0.1 M tris(hydroxymethyl)aminomethane (pH 8.0) buffer. Cells were then suspended in 1 ml of this buffer and disrupted by sonic treatment. The extract was clarified by centrifugation at 27,000 × *g* for 15 min. The extract was used either directly or following removal of salts and small molecules with a 4-ml Sephadex G-25 column. The reaction mixture contained: 0.2 ml of buffered dye mix (1), 0.2 ml of PCA (16 mM, pH 7), and 0.1 ml of extract. PCA was generously donated by Annette Baich. After incubation at 37°C for various times, the reaction was stopped by addition of 0.1 ml of 0.5 M HCl. Optical density was measured at 520 nm.

For identifying strains carrying the regulatory mutant *putC900*, a spot test for proline oxidase was used. This test is similar to the one described by Dendinger and Brill (7). Colonies were patched onto glass petri dishes containing succinate-ammonium medium and grown for 24 h. One milliliter of toluene was added to the lid of an inverted dish and incubated 10 min at 37°C to permeabilize cells. The patches were then covered with 9.0-cm disks of Whatman no. 1 filter paper soaked with 0.6 to 0.7 ml of a solution containing 3 M proline, 0.05 M *o*-aminobenzaldehyde, and 100 μg of chloramphenicol per ml. The plate was then incubated at 37°C for 15 to 30 min. Patches of cells carrying the *putC* mutation turn bright yellow; other colonies remain pale.

RESULTS AND DISCUSSION

Mutant isolation. The availability of positive selections for *put* mutants makes it possible to easily select large numbers of spontaneous mutations. Approximately 10 to 20% of spontaneous mutations are deletions (10, 20); this has enabled us to collect a large number of deletion mutants for use in construction of a detailed genetic map of the *put* region.

Table 2 presents results of the positive selections used. All Put⁻ mutants selected using AC or DP proved to be defective in permease. These permease mutations mapped near *pyrC* and *pyrD*, as do mutations affecting proline degradative enzymes. This suggested the existence of a gene cluster encoding both the permease and the enzymatic activities. Further evidence for this gene cluster is the finding that among the deletion mutants selected for permease defects were some mutants which also lacked the proline degradative activities. Apparently single deletion mutations can affect both the permease gene(s) and the genes encoding the degradative enzyme structure.

The percentage of AC-resistant mutants that were Put⁻ was strongly increased if the parent strain carried the regulatory mutation, *putC900*. Such strains grew with high levels of permease and could achieve higher concentrations of the analog. Apparently there is a class of mutations not leading to a Put⁻ phenotype which can cause resistance to the AC levels achieved by *put*⁺ cells. Mutations of this class only rarely provide resistance to AC in constitutive (*putC*) strains. Therefore, when the *putC* mutant was used as parent, 98% of AC-resistant mutants were *Put*⁻; over half of these mutants proved to be deletions. Apparently, in the constitutive strain only mutations which caused a drastic reduction in permease level were able to provide AC resistance.

Mutants resistant to DP were almost exclusively Put⁻. This analog was more toxic than AC; apparently the sort of mutation arising in *putC*⁺ cells which provided AC resistance without a defect in proline utilization cannot provide resistance to DP.

Baikiain was the least toxic of the three proline analogs. As first shown by Dendinger and Brill (8), this analog inhibits only strains carrying the *putC* regulatory mutation; since many of the baikiain-resistant mutants retain an intact permease, it seems that some other function in the *put* region is also essential to toxicity. This essential function is probably proline oxidase, since many baikiain-resistant mutants lacked this activity. In addition, Dendinger and Brill (8) showed that baikiain serves as a substrate for this enzyme. Their suggestion that baikiain is converted to a toxic compound by proline is consistent with the mutant isolation data.

A series of point mutations defective in proline

TABLE 2. *Selection of put mutants*

Parent[a]	Analog	Percent of resistant mutants which are Put⁻	Percent of Put⁻ mutations affecting permease	Put⁻ mutant types	
				Revertable (%)	Stable (%)
putC⁺ (LT2)	AC	50	100	93	7
putC (TR2139)	AC	98	100	53	47
putC⁺ (LT2)	DP	98	100		
putC (TR2129)	Baikiain[b]	100	70		

[a] The regulatory mutant *putC* was isolated and described by Dendinger and Brill (7, 8). This mutant possesses high constitutive levels of proline permease and degradative enzymes even when grown in the absence of exogenous proline.

[b] Selection for baikiain resistance depends upon use of strains carrying a *putC* mutation.

utilization was sought following penicillin enrichment. These hunts were largely unsuccessful when applied to wild-type *Salmonella*, but yielded *put* mutants when the *putC* regulatory mutant was used as parent strain. However, if wild-type strains were induced by growth on proline as sole nitrogen source before mutagenesis, *put* mutants were also obtained. It seems that either preinduction enhances killing of *put*$^+$ cells in penicillin or the *put* region is more highly mutable when derepressed. Preliminary reconstruction experiments and reversion tests of various *put* mutations suggest that the latter explanation is correct (B. Ratzkin, unpublished data).

An additional method of *put* mutant selection employed an unusual auxotrophic strain isolated by Miklavz Grabnar and characterized by Rolf Menzel. The strain (TR1995) requires proline at an exogenous concentration of 8 to 10 mM. (Standard proline auxotrophs grow well on 0.2 mM proline.) The requirement for a high concentration of proline is due to the possession by TR1995 of three mutations. A deletion mutation (*proAB47*) removes two *pro* biosynthetic genes and causes auxotrophy. A second mutation (*putP639*) eliminates the major proline uptake system. A third mutation (*proP673*) presumably removes a minor, high-affinity proline uptake system. All three mutations are essential to the requirement for high exogenous proline. Strain TR1995 was transduced with phage grown on a wild-type donor, selecting for recombinants that can satisfy their proline requirement with a low (0.2 mM) concentration of exogenous proline. Many of the recombinants resulted from introduction of a wild-type *putP*$^+$ gene from the donor. If donor phage had been mutagenized, each of these transductants inherited, along with the *putP*$^+$ gene, a highly mutagenized *put* region. Among these *putP*$^+$ transductants, mutants with lesions in the degradative enzyme gene (*putA*) were recovered at a frequency of 10 to 15%.

Mutants. A summary of the mutants isolated, their source, and their properties is included in Table 3. Isolation methods are numbered in the table and refer to numbered procedures de-

TABLE 3. *Source and characteristics of put mutations*

put alleles	Mutation type[a]	Mutagen used to induce[b]	Selection method[c]	Affected function[d]
500	S	DES	1	po
513, 527, 530, 558, 584	S	Sp.	1	po
704	S	Sp.	3	o
515–518, 522–524, 534, 540, 549–551, 555, 557, 559, 563, 567, 570, 572, 586, 587, 590, 594	D	Sp.	1	po
679, 715	D	Sp.	3	po
521, 538, 544, 552, 553, 560, 574, 579, 581, 588, 593, 598	N	Sp.	1	po
600	Pt.	Sp.	1	p
604	Pt.	Sp.	1	po
609	Pt.			o
647, 690–695	Pt.	Sp.	3	o
601	Pt.	DES	1	po
638, 639	Pt.	DES	7	p
649, 653	Pt.	DES	5	p
650–652, 654, 655	Pt.	DES	5	o
616, 618, 619, 621–623	Pt.	NG	1	p
617	Pt.	NG	1	po
731–733, 735–745, 748	Pt.	HA	6	o
734, 746, 747	Pt.	HA	6	p
645, 646	Pt.	ICR191	2	p
610	F	ICR191	4	po
611–615, 624	F	ICR191	4	o
656–665, 668, 669	F	ICR191	1	p
666, 667	F	ICR191	1	po
670, 696–701	F	ICR364OH	4	o
625, 626, 630	F	DES	4	o
631–637	T	DES	1	p

[a] S, Stable, no revertants detected; Pt., induced to revert by NG; D, demonstrable deletion; N, nontransducible deletion; F, frameshift, induced to revert by ICR191; T, temperature sensitive.

[b] Sp., Spontaneous; DES, diethyl sulfate; NG, nitrosoguanidine; ICR, acridine derivative ICR191 or ICR364OH; HA, hydroxylamine.

[c] Refer to numbered procedures in the text.

[d] p, Permease; o, oxidase and/or dehydrogenase.

scribed in Materials and Methods.

Map. A genetic map of the *put* region is presented in Fig. 1. This map was constructed by transductional crosses using various deletion mutations as recipients and point mutants as donors. A point mutant was considered to lie within the region of the map deleted in the recipient strain if no recombinants were seen in crosses which would have yielded more than 5,000 recombinants with a wild-type (*put*$^+$) donor. Recombination between point mutations was scored as positive if greater than 0.1% recombination was seen. The sensitivity of these crosses is low since the *put* region is rather poorly transduced by P22.

Analog-resistant mutants that were defective in permease activity clustered at the left side of the region as drawn (Fig. 1). This region has been designated *putP*. All of the *put* mutants lacking the ability to degrade proline but retaining permease activity (and sensitive to AC inhibition) mapped at the right side of the map. As will be discussed later, we believe that this region codes for a single bifunctional polypeptide which has both proline oxidase and PCA dehydrogenase activities. This region has been designated *putA*. The regulatory mutation *putC900* seems to be located between the two structural genes. Each of the three regions will be discussed in more detail below.

Permease region (*putP*). At present this region is defined only by genetic mapping of mutants defective in permease. Since no complementation tests have been done, we do not yet know how many cistrons might be located in this region. By analogy with the histidine permease region, which contains at least three functional units (2), it would not be surprising if the *putP* region proved to include more than one cistron.

Structural gene for proline degradative enzymes (*putA*). A single gene (*putA*) seems to encode the structure of both proline degradative enzymes, proline oxidase and PCA dehydrogenase. A single bifunctional polypeptide produced by this gene possesses both catalytic activities. Several lines of evidence support this conclusion. Almost all mutants mapping in the *putA* region lacked both oxidase and dehydrogenase (see Table 4). Only two exceptional mutants have been found which lack only one of the two activities. These mutants (*put-601* and *put-609*) are also presented in Table 4. We feel these will prove to be missense mutations affecting only one of the catalytic activities of the bifunctional protein. In Table 4, deletion mu-

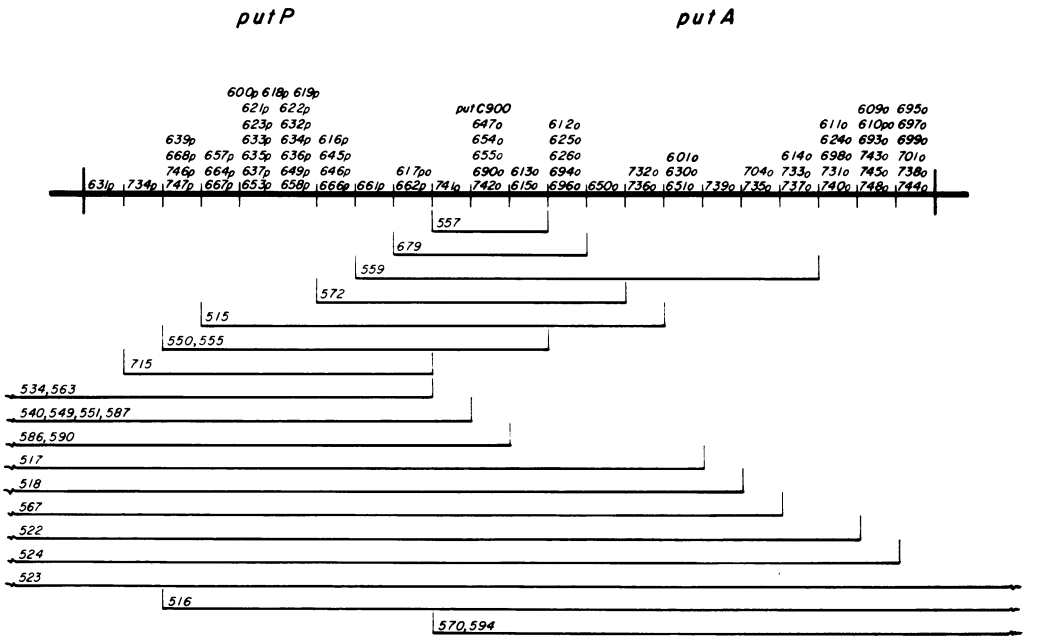

FIG. 1. *Genetic map of the put region. Point mutations are presented above the heavy horizontal line. Those mutants designated "p" are assumed to lack permease based on either assay of proline uptake or AC resistance. Mutants designated "o" are assumed to be defective in proline oxidase and/or PCA dehydrogenase activity. This is based either on enzyme assay or on failure to grow on leucylproline as sole nitrogen source (see text for details). The regulatory mutation is located roughly in the center of the map. It is not yet clear where in this region the boundaries between genes and regulatory region lie.*

TABLE 4. *Enzyme activities in putA mutants*[a]

Mutant type	Relevant genotype	Strain designation	PCA dehydrogenase	Proline Oxidase
Parental strains	*put*$^+$	LT2	100	100
	put$^+$	pyrC7	100	100
putA deletion mutants	Δ*put-570*	TR4957	14	5
	Δ*put-594*	TR4981	24	3
	Δ*put-559*	TR4946	1	3
Typical *putA* point mutants	*putA610*[b]	TR3590	<1	3
	putA617	TR3595	7	3
	putA625		2	3
	putA626		2	3
	putA630		4	3
Exceptional *putA* mutants	*putA601*	TR3557	88	5
	putA609	TR2140	13	100

[a] Enzyme activities of mutants are expressed as percentages of the level found in *put*$^+$ control strains grown and assayed under the same conditions as the mutant strain. All cells were grown on succinate-ammonia medium with 0.2% proline present as inducer. Considerable variation in PCA dehydrogenase levels is observed. A high nonspecific background dehydrogenase activity is seen due to reduction of dyes by reactions not dependent on the presence of substrate PCA. For a *put*$^+$ strain, a typical value of 12 U (change in optical density at 520 nm per hour per milligram of extract) is seen, while *putA* mutants show values of around 2 to 4 U due to the nonspecific reactions. The background level can be reduced by removing endogenous substrate from extracts by Sephadex G-25 chromatography. This reduces the background activity, but also causes a reduction in PCA dehydrogenase activity. The proline oxidase assay gives more consistent results. The oxidase level seen in *put*$^+$ control strains was 1.1 U (change in optical density at 440 nm per hour per optical density at 650 nm of toluenized cells) when assayed as described in the text.

[b] Equivalent data were obtained for mutants *put-611–615* and *put-624*.

tants *put-570, -594,* and *-559* show considerable residual activity. These strains were isolated in a different genetic background from the other strains presented. The apparent residual activity seen is likely to be due to differences in the levels of nonspecific dehydrogenase activities. Definitive comparison of deletion and point mutants must await construction of isogenic strains.

Complementation tests of *putA* mutations will shed more light on the nature of this region. These tests will require a F′ episome carrying the Salmonella *put* locus. Direct evidence for the existence of a bifunctional protein is the observation that both activities copurify and the purest preparations contain a single polypeptide of 135,000 molecular weight. These data will be presented in detail later (Rolf Menzel, manuscript in preparation).

***putA* and *putP* genes are in separate operons.** A series of frameshift mutants of *putA* and *putP* was checked for evidence of polarity effects. These mutations were all induced by the frameshift mutagen ICR191; they are all induced to revert by ICR191 and by hycanthone, another frameshift mutagen (11). Frameshift mutations are known to show polar effects on expression of promoter-distal genes in the same operon (15). All *putA* frameshift mutants tested showed sensitivity to inhibition by AC; all *putP* mutants utilize the proline in leucylproline as sole nitrogen source. Thus, there was no obvious evidence of polar effects.

This conclusion was confirmed by the enzyme assays; only slight if any polarity effects were seen (Table 5). Frameshift mutations in either *putA* or *putP* failed to show polarity effects on expression of the other gene. This strongly suggests that these two genes are in separate transcription units. It should be mentioned that while the frameshift mutations tested above showed no evidence of polarity, one exceptional mutant was isolated following ICR mutagenesis. It lacked both permease and oxidase and will be discussed later.

Control region (*putC*). Between the *putP* and *putA* genes lies a region which seems to include regulatory elements. This control region contains two sorts of mutations which have properties characteristic of regulatory defects. The first is the *putC900* mutation, described by Dendinger and Brill (7). This mutant produces high levels of both permease and degradative enzymes even in the absence of the inducer, proline. Since the permease and the degradative enzyme seem to be produced by different operons (see above), the *putC* mutation must be involved in regulation of more than one unit of transcription. Preliminary complementation

TABLE 5. *Absence of polar effects of frameshift mutations in the putA and putP genes*[a]

put mutation	Strain no.	Proline oxidase	Proline permease
put$^+$	LT2	100	100
putA611	TR3591	5	100
612	TR3592	5	94
614	TR3593	5	120
615	TR3403	5	111
625		5	76
putP657		100	8
658		100	4
659		89	4
664		100	2
667		120	3
670		89	10
Δ(*putA-P*)*500*		5	1

[a] Cells were grown in succinate-ammonium medium containing essential supplements and 0.2% proline as inducer. Activities are expressed as a percentage of the level seen for strain LT2. This control strain has a proline oxidase level of 0.66 μmol/h per unit of optical density at 650 nm of cells when assayed as described in the text; its permease level is 240 pmol/min per unit of optical density at 650 nm of cells. Permease was assayed following starvation of cells as described in the text.

data using an *E. coli* F′ episome show that *putC900* is recessive and may code for a diffusible repressor protein; alternatively, the *putC* mutation could affect a site acting *cis* to regulate transcription both the left (*putP*) and right (*putA*). Further complementation tests with homologous episomes should help decide between these alternatives.

The map position of *putC900* was determined initially by deletion mapping. *put* deletion mutants, isolated in a parental strain which possesses the *putC900* mutation, were used as recipients. Unless removed by the deletion, the *putC900* mutation should be present in all of these strains. Phage grown on a wild-type (*put*$^+$) strain was used for transductional crosses with this series of deletion mutants. Put$^+$ recombinants were selected and screened for evidence of recombinants which had retained the recipient regulatory mutation. Existence of such a recombinant would indicate that *putC900* and the particular deletion did not overlap.

In cases where all recombinants are *putC*$^+$, it is inferred that the *putC* site must have been removed by the deletion mutation. This method was used for seven deletion mutations (*put-555, -563, -570, -586, -590, -594,* and *-679*). Only deletion *put-563* gave Put$^+$ recombinants carrying the *pyrC900* mutation. This suggested that all deletions except *put-563* crossed the *pyrC900* site. This conclusion was only tentative, since relatively few recombinants (50 to 300) were tested and rare *putC900* recombinants could have been missed.

The resolution of this mapping method was increased by using donor strains carrying *put* point mutations. In these crosses, donor-type recombinants were counterselected, thereby increasing the likelihood of recovering rare recombinant types. Two examples of these crosses are shown in Fig. 2.

For this test, recipient strains carried *put* deletion mutations which had been isolated from a *putC900* parent. Donor strains carried an appropriately chosen *put* mutation mapping outside but near the end of the deletion. Selection was made for Put$^+$ recombinants; *putC900* was again the unselected marker. Each recombinant resulted from an exchange within the region between the donor's point mutation and the recipient's deletion end points. Donor-type recombinants were counterselected by this configuration. If the recipient's *putC900* mutation is present in this region, a higher percentage of recombinants will inherit the *putC* lesion than is seen in the cross in which the donor was *put*$^+$. Fifteen deletions were used in these crosses. Data are presented in Table 6, and the position of *putC900* inferred from these crosses is presented in Fig. 1. The effect of the donor *put* mutation on the frequency of *putC900* recombinants was as expected. For example, in earlier crosses (Δ*put-563 putC900* × *put*$^+$ *putC*$^+$), only 1% of the Put$^+$

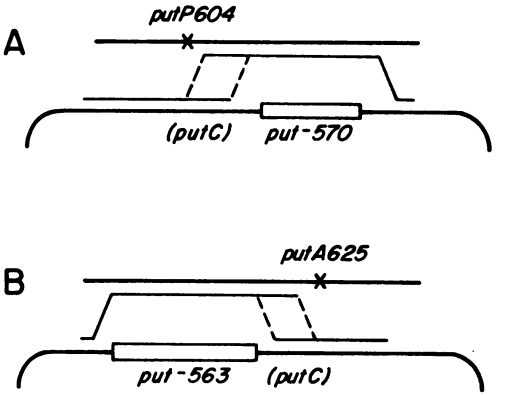

FIG. 2. *Design of crosses performed to detect recombination between deletion mutations and putC900. Recipients used are deletion mutants isolated from a strain carrying putC900. Each deletion strain was crossed with a donor carrying a put point mutation mapping just to the left (A) or right (B) of the deletion in question. Put*$^+$ *recombinants were selected and scored for inheritance of the recipient putC900 mutation. If the deletion crosses the site of putC900, no such recombinants should be recovered. If the deletion lies outside of putC900, one of the two arrangement (A or B) should yield putC recombinants.*

TABLE 6. *Deletion mapping of putC900*[a]

Deletion mutation	Donor point mutation	Inheritance of *putC900*
put-557	*put-661* (L)	0/34
put-679	*put-609* (R)	0/50
	put-639 (L)	0/50
	put-661 (L)	0/34
put-559	*put-600* (L)	0/50
	put-639 (L)	0/50
put-715	*put-655* (R)	31/34
put-534	*put-655* (R)	24/34
put-563	*put-655* (R)	3/34
put-540	*put-655* (R)	17/34
put-549	*put-655* (R)	13/34
put-551	*put-655* (R)	16/34
put-587	*put-655* (R)	13/34
put-586	*put-625* (R)	0/39
put-590	*put-625* (R)	0/34
	put-630 (R)	0/50
put-570	*put-600* (L)	0/50
	put-639 (L)	0/50
	put-661 (L)	0/34
put-594	*put-600* (L)	0/50
	put-639 (L)	0/50
	put-661 (L)	0/34

[a] The design of these crosses is presented in Fig. 2. All deletion mutations (column one) were isolated from a parent strain carrying the *putC900* mutation. The donor *put* point mutants (column two) carry no *putC* mutation. Recombinants selected (Put$^+$) all have resulted from a recombinational event between the point mutation and the end of the deletion. In column two, (L) and (R) indicate whether the point mutation is at the left (L) or right (R) side of the deletion in question. Inheritance of the *putC900* mutation was scored by the plate method described in Materials and Methods. Recombinants carrying the *putC* mutation are only detected if recombination occurs between the deletion and *putC900*; if the recipient deletion removes the *putC* side, no *putC900* recombinants can arise.

recombinants retained *putC900*. In the second series of crosses involving deletion Δ*put-563*, the donor carried mutation *putA625* (see Fig. 2B). In these crosses, 60% of the Put$^+$ recombinants carried the recipient *putC900* mutation. Thus, the sensitivity of the test for detection of *putC* recombinants is greatly increased.

To confirm the position of *putC900*, a series of donor point mutations was crossed with a recipient carrying both the Δ*put-563* deletion and the *putC900* mutation (see Fig 2B). Selection was made for Put$^+$ recombinants, and these were tested for inheritance of *putC900* (Table 7). For donor mutations rather far to the right of deletion Δ*put-563*, approximately 30% of the recombinants inherited *putC900* (last 15 lines of Table 7). For *put-741*, which was shown by deletion mapping to lie to the left of *putC900*, approximately 90% of the recombinants inherited *putC900*. It is concluded that *putC900* lies just to the right of *put-741* and to the left of all other mutations used as donor lesions.

The map position of *putC900* is thus roughly in the center of the whole *put* region. Most of the permease-defective mutants map to the left and most oxidase-dehydrogenase mutants map to the right of *putC900*. It can be seen in the map, however, that one oxidase-dehydrogenase mutation (*put-741*) lies to the left of *putC900*. It is possible that *put-741* will prove to be a promoter-like defect in the control region. Alternatively, the regulatory mutation may prove to lie within the oxidase-dehydrogenase gene.

Pleiotropic mutations. Two exceptional mutations caused loss of both permease and proline degradative enzyme activities. One of these mutations (*put-617*) mapped near *putC900* in the region thought to contain control elements. However, the other mutation (*putA610*) was located at the right end of the *putA* region (see Fig. 1). We have no explanation for this mutation; its phenotype and map position have been extensively checked. One possibility is that the permease and oxidase-dehydrogenase proteins may exist as a complex; particular mutations in either protein might cause inactivation of both activities of the complex. The fact that *putA610* was induced by ICR191 and thus may be a frameshift mutation makes this hypothesis less likely. However, ICR191 is a relatively weak mutagen, and some of the mutants isolated following ICR mutagenesis could have arisen as spontaneous events. Further characterization of this mutation should resolve these questions.

Map position of the *put* genes. The *put* gene cluster was localized by preliminary Hfr

TABLE 7. *Three-point crosses to map putC900*[a]

Donor point mutation	Percent of Put$^+$ recombinants inheriting recipient *putC900* mutation
put$^+$	4 (7/184)
put-741	90 (19/21)
put-654	20 (10/50)
put-742	56 (20/36)
put-625	54 (98/182)
put-626	51 (51/100)
put-732	24 (12/50)
put-630	26 (39/150)
put-651	31 (11/36)
put-624	35 (52/150)
put-740	31 (5/16)
put-609	30 (61/200)
put-743	36 (18/50)
put-745	27 (7/26)
put-748	14 (7/50)
put-738	4 (2/50)
put-744	24 (12/50)

[a] Recipient was Δ*put-563 putC900*.

crosses using the AC resistance mutation *putP500*. These crosses indicated a map position near the *pyrC* gene at min 22 of the revised *Salmonella* chromosomal map. This location was confirmed by transductional crosses using both P22 and P1 transducing phages (Table 8). Condamine (5) has demonstrated linkage between the gene for proline oxidase and the *pyrC* locus in *E. coli*.

The data in Table 8 suggest that the *put* genes might be located between *pyrC* and *pyrD*, since the *pyrC* and *pyrD* loci are not cotransduced by P22 and are only weakly cotransduced by P1; 9% linkage was seen by Christoph Beck (personal communication). This location of *put* was confirmed by transductional crosses using large deletions of the *put* region. If *put* lies between *pyrC* and *pyrD*, then strains carrying an extremely large *put* deletion (described later) might have lost sufficient material from the region between *pyrC* and *pyrD* to permit cotransduction of the two *pyr* genes by P22. Crosses were performed using a donor which carries both a large *put* deletion (*Δput-521* or *Δput-544*) and a temperature-sensitive *pyrC* mutation (*pyrC1502*). The recipient was *pyrD13*. Selection was made for inheritance of $pyrD^+$, that is, for growth on minimal medium at 30°C. Recombinants were scored for inheritance of the donor *pyrC* mutation by checking for those which required uracil at 40°C. Results show that when the donor carried either large *put* deletion, *pyrD* and *pyrC* were cotransducible by P22 at a frequency of 2%. This argues strongly that the *put* region is located between *pyrC* and *pyrD*.

Orientation of the *put* gene cluster. Three-point crosses were performed in order to orient the *put* gene cluster with respect to the outside *pyrC* gene. The double mutant *Δput-515 pyrC7* was used as recipient in transduction crosses. Selection was made for Put^+ recombinants; these recombinants were scored for inheritance of the donor pyr^+ allele. When the donor carried no *put* mutation and thus no restriction was placed on the number of pyr^+ recombinants, pyr^+ recombinants were seen at a level of 0.8% (3/400). Approximately the same percentage (1%, 8/800) of the recombinants were pyr^+ when the donor carried the *putA624* mutation, suggesting that *pyrC* and *putA* are at opposite ends of the recipient deletion. However, when the donor carried a *putP639* mutation, no pyr^+ recombinants were detected (0/600), suggesting that the *putP* mutation lies between *pyrC* and the recipient deletion; formation of such a pyr^+ recombinant would require four crossover events. These data, taken together with the data on large *put* deletions, leads us to infer the gene order *pyrC putP putA pyrD*.

Large deletions of the *put* regions. In screening deletion mutations which affect the *put* region, many were encountered which removed the entire *put* region and large amounts of adjacent material. The large size of these deletions was first indicated by the failure of P22 to transduce a large enough fragment to correct their defects. These large deletions were common; of 83 deletions isolated, 13 were not repairable by transduction. These deletions are listed in Table 3 with the designation N (nontransducible). The strains were transducible at other loci, but showed no Put^+ transductants in tests that would have yielded 10^3 Put^+ transductants had a *put* point mutant been used as recipient. Results outlined below suggest that between 40 and 117 kilobases (kb) of DNA was removed by such deletions and that no essential genes were located close to the *put* region. None of the deletions extended into the *fabA* or *-D* genes, which were mapped in this region by Semple and Silbert (21).

The conclusion that the nontransducibility of these deletions is due to their large size is based on the following lines of reasoning: (i) one of the deletions (*Δput-544*) was found to be transducible by phage P1, which is over twice as large as P22; (ii) in deletion strains, linkage between *put* and the neighboring *pyrC* and *pyrD* genes was markedly increased (see Table 9); (iii) the *pyrC* and *pyrD* loci, which bracket the *put*

TABLE 8. *Transductional linkage of put and pyrC*[a]

Recipient	Selected Phenotype	Percent contransduction by P22 of indicated mutations		Percent contransduction by P1 of indicated mutations		
		putP500	*putP608*	*putP500*	*putP608*	*putA609*
pyrC7 (TR2230)	Pyr^+	1.8 (220)	1.9 (408)	52 (97)	42 (50)	26 (50)
pyrD13 (TR2228)	Pyr^+	<1 (100)		8 (104)	12 (100)	8 (100)

[a] Recombinants (Pyr^+) were selected on minimal medium; histidine was included to satisfy a nutritional requirement of one of the recipient strains. Recipient strains carry the *galE* mutation. For crosses using P1 phage, recipients were pregrown on glucose and thus were sensitive to P1 infection. When P22 was used, recipients were pregrown on glucose plus galactose and thus were sensitive to P22 infection. The various *put* alleles tested were carried in the donor strain. The number of transductant clones scored is presented in parentheses.

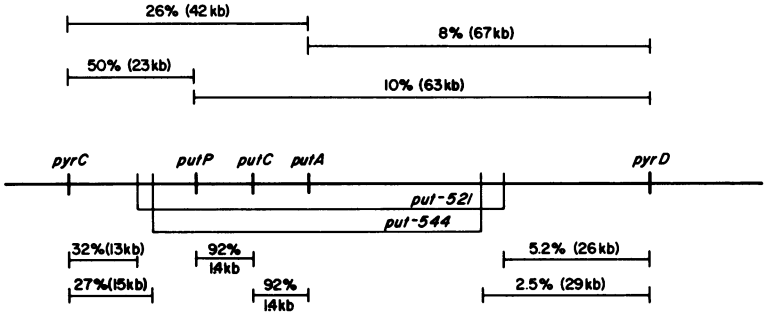

FIG. 3. *Size of the put region estimated by transduction. Percentages presented above the map are P1-mediated cotransduction frequencies; those below the map are from P22-mediated crosses. Cotransduction frequencies were converted to physical distance by the formula of Wu (25).*

TABLE 9. *Effect of large put deletions on linkage to pyrC and pyrD*

Donor mutation	Mutation type	Percent of Pyr+ transductants receiving donor *put* mutation[a]	
		Recipient *pyrC7*	Recipient *pyrD13*
putC900	Point mutation	0.8 (3/400)	<0.2 (0/500)
put-532	Small deletion	0.8 (3/400)	<0.2 (0/500)
put-544	Large deletion	27 (106/400)	2.5 (10/397)
put-521	Large deletion	32 (63/200)	5.2 (18/349)

[a] All of these crosses are P22-mediated transductional crosses.

region, are normally not cotransducible. In strains carrying large *put* deletions, linkage between *pyrC* and *pyrD* was detectable (see previous section).

Size of the *put* region. Very rough approximations of the size of the *put* region can be made by using two-point cotransduction frequencies. These estimates are not highly reliable due to marker effects of unknown origin (9). However, by using the relationship suggested by Wu (25) for converting cotransduction frequency to physical size, one can make this rough estimate. Uncertainties are somewhat lessened by averaging several estimates for the same interval. For these calculations, the P22 genome size is based on measurement by Rhodes et al. (17) and Tye et al. (23).

Two-point linkage data suggest that the average of several distances calculated from *putC900* to several *putA* mutations is 1.4 kb; the average distance of two *putP* mutations to *putC900* is also 1.4 kb. This suggests that the distance from the middle of *putP* to the middle of *putA* is of the order of 3 kb. If a typical gene is of the order of 1 kb in size, then the control region is small but could include structural genes for one or two additional proteins.

The size of two large deletions and the distance from *put* to the neighboring *pyrC* and *pyrD* genes can also be estimated crudely by converting cotransduction frequencies to physical distance. A summary of such data is presented in Fig. 3. All distances present in the figure should be regarded as approximate due to the many problems associated with two-point mapping.

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