PROLINE MUTANTS OF SALMONELLA TYPHIMURIUM[1]

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ACCORDING to previous analyses of 16 proline-requiring mutants of *Salmonella typhimurium*, made genetically by NIELSEN (DEMEREC *et al.* 1954) and biochemically by KANAZIR (1956; DEMEREC *et al.* 1955), the mutants fell into at least three groups. Recently, the number of proline-requiring mutants in our collection was increased to more than 200, and useful new techniques were devised or adapted for their study, such as abortive transduction (OZEKI 1956) and recombination between *S. typhimurium* and *Escherichia coli* (BARON, SPILMAN and CAREY 1959; MIYAKE and DEMEREC 1959; MIYAKE 1959; MIYAKE, unpublished). These developments justified further investigations of the proline mutants, which will be reported here.

MATERIALS

The mutants were isolated, by several investigators in M. DEMEREC's laboratory, from the following prototrophic *S. typhimurium* strains: wild type strain LT-2; wild type strain LT-7; and LT-2 (or LT-7) in which a part of the chromosome in the *pro* region had been replaced by a homologous portion from strain LT-7 (or LT-2) through transduction with mutant H4 of phage PLT22. Most of the mutations to proline requirement occurred spontaneously; a few were induced by irradiation with ultraviolet light. Selection was by the standard penicillin screening procedure.

Bacterial mutants are referred to by the symbols proposed in Microbial Genetics Bulletin, No. 16 (1958): *pro* means proline requirement; *glt*, glutamic acid requirement; *cys*, cystine requirement; *lac*$^+$ and *lac*$^-$, ability and inability to utilize lactose.

The bacteriophage in most of the experiments was temperate phage PLT22 H1 (ZINDER and LEDERBERG 1952). Mutants H4 and H5 of the same strain were used to obtain sensitive transductants and to test lysogenicity of bacteria, respectively.

The minimal agar medium for the transduction experiments contained K_2HPO_4, 10.5 gm; KH_2PO_4, 4.5 gm; $(NH_4)_2SO_4$, 1.0 gm; sodium citrate, 0.97 gm; glucose, 2.0 gm; minimal agar, 15.0 gm; demineralized water, 1000 ml. In some experiments the minimal medium was enriched with 0.01 percent dehydrated nutrient broth, to bring about a higher frequency of transduction. When *lac*$^+$

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was the selective marker, sodium citrate was omitted from these media.

The nutrient broth medium in which bacteria were grown or phage stocks prepared contained: NaCl, 4 gm; nutrient broth, 8 gm; demineralized water, 1000 ml. Nutrient agar medium for bioassay of bacteria or phage contained: NaCl, 5 gm; nutrient agar, 23 gm; demineralized water, 1000 ml. EMB agar medium, on which bacteria were tested for sugar fermentation ability, contained tryptone, 10 gm; yeast extract, 1 gm; NaCl, 5 gm; K_2HPO_4, 2 gm; eosin Y, 0.4 gm; methylene blue, 0.065 gm; agar, 15 gm; sugar, 10 gm; demineralized water, 1000 ml.

In supplementing minimal medium with an amino acid, 20 mg was added to 1000 ml of medium.

EXPERIMENTAL RESULTS

Analysis by transduction: For transduction tests, 1 ml of fresh saturated bacterial culture grown in nutrient broth (approximately 2×10^9 bacteria per ml) was mixed with 0.1 ml of phage suspension of T2 buffer containing about 10^{11} phage particles per ml, so as to have a multiplicity (phage/bacterium) of five, and then kept at 37°C for 6–8 minutes while adsorption took place. The mixture was then plated on either minimal or enriched minimal agar, 1/10 ml per plate. Colonies were scored after 24–48 hours of incubation. Large colonies appeared as the result of complete transduction; and minute colonies, which were generally five to 15 times more numerous, resulted from abortive transduction. The fact that the small colonies did originate by abortive transduction was verified by means of OZEKI's (1956) adaptation of NEWCOMBE's respreading technique. The test was made with *proA-15* and wild type phage, and gave positive results. The presence of minute colonies in a transduction experiment involving two phenotypically similar markers is positive evidence that they complement each other, whereas absence of minute colonies indicates noncomplementation (DEMEREC and OZEKI 1959).

The transduction experiments showed that the 127 *pro* mutants tested can be divided on the basis of complementation into four groups: *proA*, *proB*, *proC*, and *proD*. Furthermore, they revealed close linkage between mutants of groups *A* and *B*, since the number of complete transductions was consistently smaller when the experiment involved members of these two groups than when either donor or recipient belonged to another group, or when the donor was wild type. Typical results as to numbers of complete transductants and presence or absence of abortive transductants in inter- and intragroup experiments are given in Table 1. The close linkage between the *proA* and *proB* groups was confirmed by an analysis of multisite mutants, which will be discussed later. Of the 127 mutants tested, 42 are *proA*, 63 *proB*, 18 *proC*, and four *proD*.

Since *proA*, *B*, and *D* mutants can grow on medium containing glutamic-γ-semialdehyde, which does not support growth of *proC* mutants, it was possible to determine whether or not *proC* is carried in the same transducing fragments with other *pro* markers, by transduction experiments in which *proC* was recipient, any of the other mutants was donor, and the selective medium contained glutamic-

TABLE 1

Transduction analysis of proline-requiring mutants of Salmonella typhimurium

Recipients	Donors								Control
	proA-39	*proA-46*	*proB-9*	*proB-45*	*proC-90*	*proC-95*	*proD-102*	Wild type	
proA-39	—	—	+	+	+	+	+	+	—
	0	38	521	105	1,920	2,682	12,342	1,558	0
proA-46	—	—	+	+	+	+	+	+	—
	195	0	269	194	2,746	2,898	14,872	1,678	0
proB-9	+	+	—	—	+	+	+	+	—
	110	19	14	9	1,396	2,040	8,236	868	19
proB-45	+	+	—	—	+	+	+	+	—
	369	60	54	0	2,128	2,440	8,810	1,412	0
proC-90	+	+	+	+	—	—	+	+	—
	1,240	316	1,250	186	0	17	5,388	293	0
proC-95	+	+	+	+	—	—	+	+	—
	545	95	1,122	162	11	0	2,722	279	0
proD-102	+	+	+	+	+	+	—	+	—
	988	96	410	189	526	675	0	359	0

Plus and minus signs indicate presence or absence of abortive transductants. Each figure records number of complete transductants on three enriched minimal agar plates (about 2×10^8 bacteria and 10^9 phage particles per plate). Figures in control column record numbers of spontaneous mutants.

γ-semialdehyde. Colonies of *proA*, *B*, or *D* would appear only when both markers of the experiment were carried in a single fragment. The results, given in Table 2, show that *proA*, *B*, and *C* are located in the same transducing fragment. The *proD* results are negative, but the tests were not conclusive enough to justify a final conclusion.

Biochemical analysis: The pathway of proline biosynthesis has been studied by several investigators in various organisms (see VOGEL 1955). Data available at present indicate that the pathway is as follows:

glutamic acid ⟶ glutamic-γ-semialdehyde ⟶ proline

Therefore the growth responses of *pro* mutants to glutamic acid, glutamic-γ-semi-

TABLE 2

Numbers of wild type and donor type transductants observed in experiments with four proline mutants

Recipient		Donors		
		proA-15	*proB-9*	*proD-102*
proC-90	Wild	2,765	1,838	1,166
	Donor type	3	2	0

Plating on minimal medium supplemented with glutamic-γ-semialdehyde (about 2×10^8 bacteria and 10^9 phage particles per plate).

aldehyde, proline, and hydroxy proline were tested to determine the positions of the blocks for which the mutant genes are responsible. Ornithine was included in the tests because studies by KANAZIR (1956) had indicated that ornithine supports partial growth of one proline group of Salmonella mutants, but all the ornithine tests were negative.

A saturated culture grown in broth was centrifuged, washed, and resuspended in saline, and 0.1 ml of the suspension was plated on minimal agar. A piece of the agar was removed with a sterile cork borer, and 1–2 drops of an aqueous solution of a possible proline precurser (2 mg/ml) was dropped into the hole. After 18–24 hours of incubation, growth responses were examined. The results are summarized in Table 3. They show clearly that in *proA* and *proB* mutants the biosynthesis of proline is blocked somewhere between glutamic acid and glutamic-γ-semialdehyde. They do not differentiate, however, between the two groups. In 16 of the 18 *proC* mutants the requirement cannot be satisfied by glutamic-γ-semialdehyde; therefore, the *proC* block appears to be between glutamic-γ-semialdehyde and proline. The other two (*proC-4* and *proC-124*) show limited growth on hydroxy proline, glutamic acid, and glutamic-γ-semialdehyde. This finding can be explained by the assumption that *proC-4* and *-124* are leaky when grown in medium containing certain intermediate compounds of proline biosynthesis. The nutritional requirement in the four *proD* mutants can be satisfied by either glutamic acid, glutamic-γ-semialdehyde, or proline, so that it appears that the *proD* mutation blocks proline synthesis before glutamic acid. None of these mutants, however, grows as well on medium containing glutamic acid or glutamic-γ-semialdehyde as it does on medium containing proline, and therefore the present determination of the position of the *proD* block should be considered tentative.

Syntrophism among the four complementation groups was studied in the following manner. Bacteria from a saturated broth culture were washed in saline and plated on minimal agar (ca. 2×10^8 cells per plate). A piece of agar was removed, 0.1 ml of a saturated broth culture of another bacterial strain was poured

TABLE 3

Growth responses of proline-requiring mutants to various related chemicals

Loci	Mutants	min*	hp	glt	gs	pro	nut
A	*proA-39*	—	—	—	+++	+++	+++
A	*proA-46*	—	—	—	+++	+++	+++
B	*proB-9*	—	—	—	+++	+++	+++
B	*proB-45*	—	—	—	+++	+++	+++
C	*proC-90*	—	—	—	—	+++	+++
C	*proC-28*	—	—	—	—	+++	+++
C	*proC-4*	—	++	±	++	+++	+++
C	*proC-124*	—	++	++	++	+++	+++
D	*proD-132*	—	—	+	+	++	++
D	*proD-133*	—	—	+	+	++	++

* min = minimal medium; hp = minimal medium supplemented with hydroxy-1-proline; glt = minimal medium supplemented with glutamic acid; gs = minimal medium supplemented with glutamic-γ-semialdehyde; pro = minimal medium supplemented with proline; nut = nutrient medium.

into the hole, and after 18–24 hours of incubation the growth response of the background bacteria was examined. It was found that *proC* feeds either *proA* or *proB*, further evidence for the conclusion that the biosynthetic block effected by mutations in the *proC* locus occurs at a step which is later than the step or steps controlled by *proA* and *B*. No other inter- or intragroup syntrophism was observed.

Multisite mutants: Most of the proline mutants studied revert spontaneously to wild type and recombine with other allelic mutants. They behave as "single-site" mutants, the result of change at a single site of a gene locus. A few, eight out of 206, are "multisite" mutants caused by mutation affecting several, presumably adjacent, sites; they do not undergo reversion. One of these (*proC-110*) covers five out of six sites of the *proC* locus that have been tested, and the other seven extend over various regions of *proA* and *B* (see Figure 1). Three of them (*proAB-47*, *-126*, and *-21*) are associated with sites of both *proA* and *proB*, and provide conclusive evidence that these two are located close together on the chromosome. This evidence, together with the results of the biochemical tests, raises the question whether *proA* and *proB* are two independent gene loci or just two complementation groups of the same locus. The question will be resolved only after the biochemical pathway of proline synthesis becomes better known. In the meantime *A* and *B* will continue to be regarded as two loci.

With the help of overlapping multisite mutants, the relative positions of certain sites of the *pro* loci were determined (Figure 1). The map is very incomplete; a complete map, which would be laborious to prepare, is not needed for the problems considered here.

An unusual and interesting type of behavior was revealed by the analysis of *proAB-47* and *-126*. These two mutants, which originated independently, resemble each other not only in covering all tested sites of loci *proA* and *B* but also in giving rise to much smaller numbers of transductants than other *proA* and *B* or *proC* mutants (Table 4). An interpretation of this behavior is suggested in the discussion.

Analysis by hybridization: It is now well established that crosses can be made between *S. typhimurium* and *E. coli* (Baron *et al.* 1959; Miyake and Demerec 1959; Miyake 1959). Because markers occupying long sections of chromosome

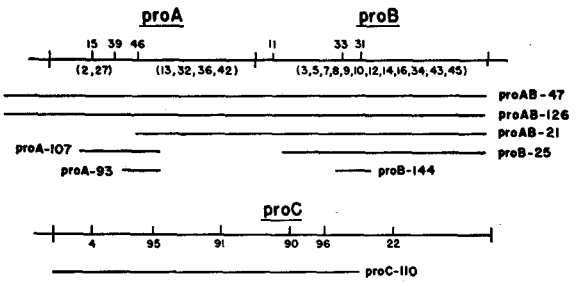


Figure 1.—Diagram indicating the extent of the deletions found in transducing fragments carrying the *proA*, *proB*, and *proC* loci.

TABLE 4

Number of transductions in experiments with two proAB *mutants and with several other* proA *and* proB *mutants*

Recipients	Donors		Control
	Wild type	*proC-95*	
proAB-47	12	26	0
proAB-126	7	9	0
proA-106	960	1,912	0
proA-109	1,088	1,500	0
proB-104	1,020	1,672	2
proB-103	864	1,752	0
proC-90	1,668	57	0
proC-22	1,856	169	5

Figures show number of transductants on four enriched minimal agar plates (about 2×10^8 bacteria and 10^9 phage particles per plate).

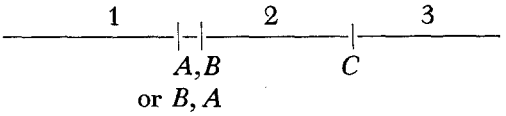
can be recombined in the hybrids, this technique is very effective for genetic mapping of the bacterial genome. It has been used in an attempt to determine the positions of three of the proline loci (*proA*, *B*, and *C*) and *glt*. The last-named locus controls a step in the synthesis of glutamic acid, which is closely related to proline synthesis; *glt* mutants grow on medium containing glutamic acid, but not on medium containing proline. Unfortunately, because the available material is not suitable, the *proD* locus could not be included in these tests.

Since in studies with *E. coli* it had been found that *pro* is located near *lac*, the experiments were made with *S. typhimurium* having the genetic constitution *lac*$^-$ *pro* (or *glt*) and *E. coli* K-12, either Hfr CS-101 (CAVALLI 1950) or Hfr H (HAYES 1953), of the constitution *lac*$^+$ *pro*$^+$ *glt*$^+$. Selection was made for *lac*$^+$, and the hybrids were tested for *pro* or *glt*. About 3000 *lac*$^+$ hybrids were tested for recombination between *lac* and *proA*, about 3000 for recombination between *lac* and *proB*, about 2000 for *lac* and *proC*, and about 300 for *lac* and *glt*. None was found, although recombination took place between *lac* and other markers involved in the tests. Extensive experiments with the hybrids (MIYAKE, in manuscript) indicated that recombination does not occur in the region carrying lactose. The most likely explanation of this finding is that Salmonella, which is *lac*$^-$, is deficient for the *lac* segment of the chromosome, and that recombination in the adjacent regions is reduced when the segment is transferred in hybrids from the *E. coli* to the Salmonella chromosome. Absence of recombination between *lac* and *proA*, *proB*, *proC*, or *glt*, then, indicates that these four gene loci are close together on the chromosome, and suggests that genes controlling related biochemical reactions are grouped together more frequently than can be detected by the transduction method.

DISCUSSION

The data presented show that *proA*, *B*, and *C* are located in the same transducing fragment, because the donor marker is recovered when *proC* serves as recipient and either *proA* or *proB* as donor (Table 2). Transduction frequencies (Table 1) reveal that *proA* and *B* are very close together, with *proC* located some

distance from them. Thus a map of the segment can be represented as shown in the diagram below.



The data of Table 2 show that in the transduction experiment *proC* × *proA* (or *B*) the number of *proA* (or *B*) transductants (recombination in regions 1–3) is very small compared with the number of wild type transductants (recombination in regions 2–3). Since it is reasonable to assume that frequency of recombinations is a function of the length of the regions within which they occur, the results suggest either that region 1 is short or that region 2 is long. If region 1 were short, one would expect considerably fewer wild type transductants in experiment *proA* × wild type, where they originate through recombination involving regions 1 and 2+3, than in experiment *proC* × wild type, where they originate by recombination in regions 1+2 and 3. The data in Table 1 show that the number of wild type recombinants is considerably larger in *proA* (or *proB*) × wild type than in *proC* × wild type, and favor the explanation that region 1 is not short but region 2 is long; they also indicate that region 3 is shorter than either region 1 or region 2.

Further light is thrown on the properties of transducing fragments carrying *pro* loci by the analysis of the two multisite mutants *proAB-47* and *-126*. Each of these mutations covers all the known sites of the *proA* and *proB* loci and behaves like a chromosomal aberration, presumably a deletion. The most interesting feature of the two mutants is that they have much lower frequencies of transduction than other *pro* mutants (Table 4). As mentioned earlier, a small number of transductants is an indication that one or both of the regions in which the responsible recombinations occur are short. The low frequencies thus suggest that the aberrations responsible for *proAB-47* and *-126* extend considerably beyond *proA* and *B*, making either one or both of the regions between the ends of the aberration and the corresponding ends of the transducing fragment very short. We can deduce, however, that only the end distant from the *proC* locus is involved, from the fact that the aberrations do not affect *proC*. This deduction is further confirmed by the results of experiments *proAB-47* (or *-126*) × wild type and × *proC-95* (Table 4), in which similar numbers of transductants were obtained with wild type and with *proC* donors. Thus the analysis indicates that mutants *proAB-47* and *-126* are due to chromosomal aberrations that begin approximately at the end of locus *proA* or *proB*, whichever is nearer to *proC*, and extend nearly to the end of the transducing fragment farthest from *proC*. Recombinations do not take place within the aberrant region. It is interesting to note that the phenotypes resulting from these two aberrations do not differ from those of single-site *proA* or *B* mutants. If the aberrations are deletions, this fact suggests that the deleted region outside *proA* and *B* either carries genes that produce no detectable effects or carries no genes at all, is genetically inert.

Among the numerous multisite mutants that have been analyzed in Salmonella only *proAB-47* and *-126* have lower frequencies of transduction than single-site

mutants of the same locus. It seems unlikely that the identification of two such rare mutations in the same region of the chromosome is due to chance. More probably, such changes occur with a higher than average frequency in this particular region. A comparable phenomenon has been observed in the *cysC* and *D* loci, where about 40 percent of the mutations belong to the otherwise rare category of multisite mutants (DEMEREC 1956).

SUMMARY

By means of genetical and biochemical tests, 127 proline-requiring mutants of *Salmonella typhimurium* were classified in four groups: 42 in *proA*, 63 in *proB*, 18 in *proC*, and four in *proD*. In the sequence of proline biosynthesis *proC* mutants are blocked between glutamic-γ-semialdehyde and proline, *proA* and *B* mutants before glutamic-γ-semialdehyde, and *proD* mutants apparently before glutamic acid. The results of transduction tests show that the *proA*, *B*, and *C* loci are carried by the same transducing fragment; *proA* and *B* are close together and *proC* is some distance from them. Hybridization experiments with *Escherichia coli* revealed that *proA*, *proB*, *proC*, and *glt* are located close to the *lac* locus.

Eight of the mutants are due to multisite mutations, presumably deletions, of which two (*proAB-47* and *-126*) cover both the *proA* and *proB* loci and extend a considerable distance toward one end of the transducing fragment. If these mutations are deletions, then the deleted region beyond *proA* and *B* either carries no genes—is genetically inert—or carries genes that produce no detectable effects.

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