THE ISOLATION AND GENETIC CHARACTERIZATION OF MUTANTS OF THE TRYPTOPHAN SYSTEM OF *BACILLUS SUBTILIS*

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IT has previously been shown in this laboratory (CARLTON 1967) and elsewhere (ANAGNOSTOPOULOS and CRAWFORD 1961, 1967) that mutations leading to defects in tryptophan biosynthesis in *Bacillus subtilis* map in a cluster on the genome of this organism, and in an order which corresponds to that obtained for similar mutants in *Escherichia coli* (YANOFSKY and LENNOX 1959) and *Salmonella typhimurium* (BLUME and BALBINDER 1966). However, the studies on *B. subtilis* reported to date have involved relatively few mutants. The work reported here describes the isolation and genetic characterization of fifty-eight tryptophan auxotrophs of *B. subtilis* induced by a variety of mutagens, including those expected to lead to frame shift mutations as well as base substitutions. The biochemical characterizations of these mutants have been reported previously (WHITT and CARLTON 1968). These mutants have been mapped within the tryptophan cluster by three-point transformation crosses and examined for their reversion properties with a variety of mutagens.

MATERIALS AND METHODS

Chemicals: ICR-170 and ICR-191, acridine half-mustard compounds, were gifts from DR. H. CREECH, Institute of Cancer Research, Philadelphia, Pa. Ethylmethanesulfonate was obtained from Eastman Organic Chemicals. Hydroxylamine hydrochloride was purchased from Matheson, Coleman, and Bell. Sodium nitrite and sodium acetate were obtained from Fisher Scientific Company. N-methyl-N′-nitro-N-nitrosoguanidine was purchased from Aldrich Chemical Co., Milwaukee, Wisconsin. 2-aminopurine nitrate, B grade, and 5-bromodeoxyuridine, A grade, were from Calbiochem. Deoxyribonuclease D was purchased from Worthington Biochemical Co., Freehold, New Jersey.

Mutant strains: The prototrophic *B. subtilis* strain SB491, derived from a transformable stock of this organism, was obtained from the Stanford University collection through the cooperation of Dr. E. W. NESTER (University of Washington, Seattle). Tryptophan auxotrophs were induced in this strain either by treating the cells directly with a mutagen, or by treatment of transforming DNA with a mutagen and recovery of new mutations by selecting for linked transformants in transformation experiments (CARLTON 1967; ANAGNOSTOPOULOS and CRAWFORD 1961).

Mutations in intact SB491 cells were produced in one of the following ways: (1) ultraviolet (UV) irradiation of cells to a survival of 10^{-4}; (2) treatment with nitrosoguanidine at a concentration of 50 μg per ml in Penassay broth for 1 hr; (3) growth of cells at 37°C overnight in Penassay broth containing 30 μg per ml of either ICR-170 or ICR-191. Appropriate dilutions of the surviving cells were plated on minimal agar supplemented with 25 μg per ml L-tryptophan, and new auxotrophs were detected by replica plating to minimal agar. Only one mutant was picked from each tube to assure the independent nature of each mutation.

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Mutations were induced in transforming DNA by one of the following procedures: *Low pH*—SB491 DNA (0.1 ml) was added to 0.1 ml of 1M sodium acetate buffer, pH 4.2, and incubated for 3½ hrs at 37°C, then diluted five-fold into 1M Tris-HCl buffer, pH 7.8.

Hydroxylamine—1.0 ml of 1.5M Na_2HPO_4 was added to 10 ml of 2.5M hydroxylamine HCl (freshly-prepared solution) in 2M NaCl. The pH was adjusted to 7.5 by addition of 6N NaOH. To 0.1 ml of this solution was added 0.1 ml of SB491, and the mixture incubated at 45°C for 30 min. At the end of the incubation period 0.8 ml of 2% acetone in minimal salts medium was added to terminate the reaction.

Ethyl methanesulfonate (EMS)—SB491 DNA was diluted to a concentration of 10 μg per ml into minimal salts medium containing 0.11M EMS, and the mixture incubated at 37°C for 3½ hrs, then diluted five-fold with a 1% sodium thiosulfate solution.

Nitrous Acid—0.1 ml of SB491 DNA was treated with 0.1 ml of freshly-prepared nitrous acid solution (0.25M $NaNO_2$ in 0.25M sodium acetate, pH 4.5) for 90 min at 25°C, then diluted five-fold with 1M potassium phosphate buffer, pH 8.5.

In these experiments the histidine marker his_2, described by NESTER, SCHAFER and LEDERBERG (1963) as being closely-linked to the tryptophan region, was utilized in the recipient strain for the recovery of newly induced mutations in transforming DNA. Cells of the his^-_2 strain were treated with 0.1 ml of the various mutagen-treated DNA preparations described above, and appropriate dilutions were plated on tryptophan-supplemented agar, which selected for transformants carrying the wild-type histidine allele carried on the transforming DNA. These transformants were replica-plated to minimal agar and all nonreplicating clones were picked, purified by two or three cycles of restreaking, and tested for growth responses to anthranilic acid and/or indole. Although these experiments were not designed to determine the quantitative levels of mutations induced by these procedures, the failure to recover tryptophan auxotrophs in control crosses with nontreated DNA indicates that these mutants were induced by the mutagenesis treatments and were not a consequence of the recombination event. We estimate from the co-transfer frequency of the his_2 marker with the tryptophan region (45–50%), that approximately half of the new mutations induced in the tryptophan region of the transforming DNA were recovered by these procedures. Induction of mutations in transforming DNA also assures that each new mutant recovered represents a different mutational event, a distinct advantage in isolating large numbers of mutants.

Each new mutant strain isolated, either from treated cells or from transforming DNA, was tested for its growth response pattern, accumulation of intermediate compounds, and stored on Difco Bacto-sporulation agar slants.

Preparation of transforming DNA: Cells used for DNA extraction were grown for 16–18 hrs in a minimal salts medium (SPIZIZEN 1958) supplemented with 0.05% casein hydrolysate (Nutritional Biochemical Corp.), 0.5% glucose, 1 μg per ml L-tryptophan, and trace elements. DNA was extracted from the cell lysates essentially by the method of NESTER and LEDERBERG (1961), except that the final solution in 2M NaCl was filtered through a HAWP 013 00 0.45 μ Millipore filter to sterilize the preparation and also to remove the majority of any denatured DNA present (WOHLHIETER, FALKOW and CITARELLA 1966). DNA concentrations were estimated by the p-nitrophenylhydrazine procedure as described by WEBB and LEVY (1958).

Mapping transformation procedures: Double mutant strains of each tryptophan auxotroph, except for the mutants in the anthranilate-responding class, were constructed with the anthranilate marker *E194* which was used as the reference marker in three-point mapping experiments (CARLTON 1966). For these constructions a double auxotroph requiring both anthranilate and histidine was used as the recipient in a transformation cross with donor DNA from each different tryptophan mutant. The transformed cells were plated on tryptophan-agar plates to select prototrophs for the closely-linked histidine marker. The transformants were replica-plated to anthranilate-agar plates and 10 clones were picked which failed to grow on anthranilate alone and had presumably acquired the donor auxotrophic marker. These presumed double mutants were first screened for the failure to accumulate intermediates in the tryptophan pathway, which provided a rapid qualitative test for the retention of the reference anthranilate marker. Non-accumulators were then tested by transformation with DNA from both the reference marker strain and from

the second tryptophan auxotroph. Failure to yield wild-type recombinants with both DNA's confirmed the double mutant nature of each strain.

In order to map mutants in the *trp E* class it was necessary to introduce a reference marker other than *E194*. For these mutants, the linked his_2 marker was introduced as the reference marker. For these constructions strain SB25 (a histidine requiring auxotroph) was used as the recipient. It was transformed with saturating levels of donor DNA from each anthranilate-requiring strain. The transformed cells were plated on minimal medium supplemented with both histidine and anthranilic acid. These plates were subsequently replica-plated to medium supplemented only with histidine and to medium supplemented only with anthranilic acid. Those colonies which did not replicate to either of the singly-supplemented media but grew on media containing both supplements were picked as double mutants, and subsequently used as recipient strains in three-point crosses.

The medium used for inducing competence in recipient strains was that described by NESTER *et al.* (1963). DNA was usually added at a concentration of 0.5 μg per ml. Cells were exposed to DNA for 20 min at 30°C with gentle shaking. The uptake was terminated by the addition of deoxyribonuclease at 10 μg per ml, and the cultures incubated at 37°C for 10 min. The cultures were then centrifuged and resuspended in 1 ml of minimal medium. For all mutants except those requiring anthranilate, appropriate dilutions were plated in duplicate on non supplemented minimal medium and on medium supplemented with anthranilic acid (10 μg per ml). Each mutant strain was tested in mapping experiments as a recipient in the double mutant configuration with the anthranilate reference marker and as a DNA donor. For crosses involving recipient mutants of the *E* class, containing the histidine reference marker, appropriate dilutions were plated on non supplemented minimal medium and on medium supplemented with histidine (20 μg per ml). Each mutant strain was tested as a recipient in the double mutant configuration with the histidine reference marker and as a DNA donor. In general, each mutant was crossed with all of the other mutants in the same phenotypic class for the determination of intragenic marker orders, and with one or two representative mutants in each of the other phenotypic classes as a check to rule out any possible aberrancies in its map position in the tryptophan gene cluster.

For the mutants in all except the *E* class the rationale for mutational site ordering was based on the relative proportions of recombinants obtained on minimal agar relative to those obtained on anthranilate agar (see Figure 1a). A donor marker was placed between the reference anthranilate marker and the recipient marker in a given cross if the frequency of fully prototrophic recombinants was less than about 35% of the total number of recombinants between the two sites being ordered, and to the right of the recipient marker if the prototrophic frequency exceeded 35%. The rationale for mutational site ordering of the *E* class mutants was based on the proportions of recombinants obtained on minimal agar relative to those obtained on histidine agar (Figure 1b). A donor marker was placed between the reference histidine marker and the recipient marker if the frequency of prototrophs was less than 35% of the total number of recombinants between the two sites being tested, and to the left of the recipient marker if the frequency of prototrophs was greater than 35%. Each cross was performed at least twice, and in some cases was repeated several times if the total number of recombinants obtained was less than 100 per plate, or if the recombinant frequencies did not permit an unequivocal order assignment for the two markers on the basis of two crosses.

Reversion studies: Each mutant was treated with each of eight different mutagens by the method of ALLEN and YANOFSKY (1963) in order to study their reversion characteristics. Single colony isolates were grown for 16–18 hrs in Penassay broth at 37°C, washed once in minimal medium, and resuspended in minimal medium. A total of about 5×10^8 cells were then spread on minimal agar plates supplemented with 0.01% casein hydrolysate and 0.01 μg per ml L-tryptophan. A sterile filter paper disc (SCHLEICHER and SCHUELL No. 740-E) was placed in the center of the plate, and one drop of the appropriate mutagen was placed on the disc. The mutagens and concentrations used were as follows: 2-aminopurine (40 mg per ml); 5-bromodeoxyuridine (8 mg per ml); hydroxylamine (0.25M); nitrous acid (0.25M); nitrosoguanidine (1 mg per ml); ICR-170 (1 mg per ml); ICR-191 (1 mg per ml); and EMS (undiluted). The plates were incubated at 37°C for five days before being read.

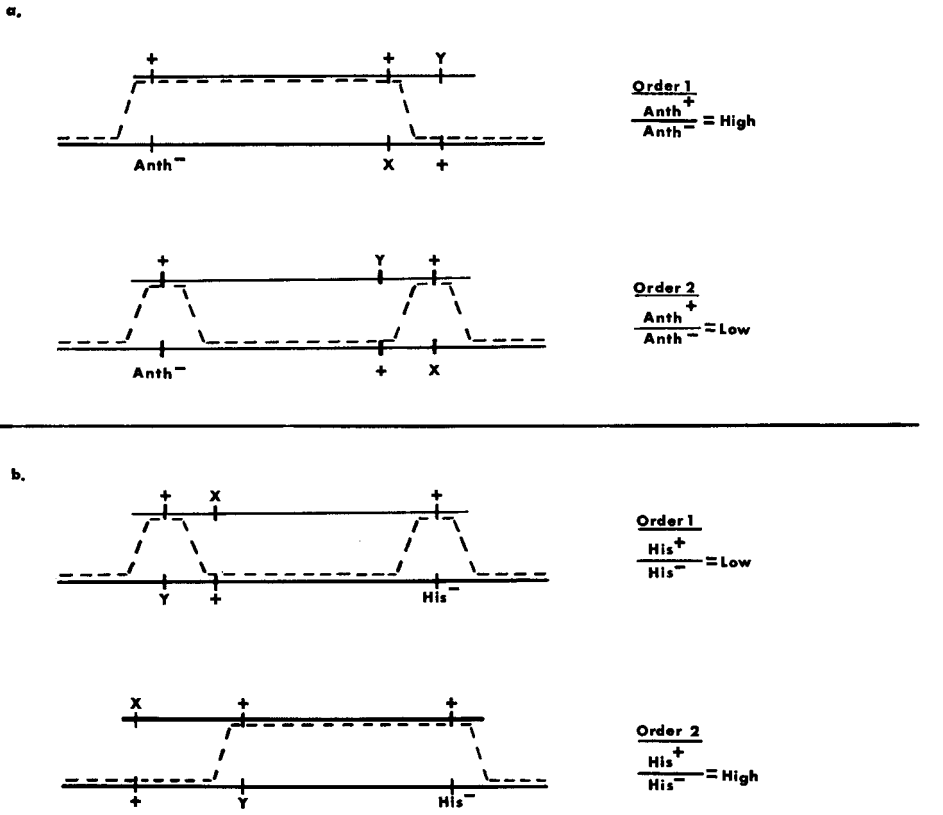

FIGURE 1.—Rationale for the determination of marker orders by three-point transformation crosses. The donor genome is represented as the shorter line and the recipient genome, carrying the reference marker, as the longer line. The reference markers used were *anth*⁻ (1a) for mapping *trp D*, *trp C*, *trp F*, *trp B*, and *trp A* mutants; and his_2^- (1b) for ordering class *trp E* sites.

RESULTS

From the mutation induction experiments a series of 58 new tryptophan-requiring mutants was recovered. The distribution of these mutants in the six phenotypic classes distinguished by growth responses and intermediate accumulations and specific enzymatic defects is presented in Table 1. In addition to these newly-isolated strains, two UV-induced strains, *trp E194* and *trp F11* (T1 and T11 of ANAGNOSTOPOULOS and CRAWFORD (1961)) and one X-ray induced strain (*trp C168*) were included from the Stanford Collection. The number of mutants induced by each mutagen in the various classes is presented in Table 2.

The results of three-point mapping experiments with the mutants are summarized in Table 3. The assignment of mutant sites to the left or right of the marker carried by the recipient was based on the average percentage of prototrophic recombinants compared to the total number of recombinants between the site in the donor and the site in the recipient, as described in MATERIALS and METHODS.

TABLE 1

Distribution of mutants into phenotypic classes

Mutant class			Mutant number	
Phenotypic*	Genotypic	Enzymatic defect	Old designation†	New designation
1	*trp E*	Anthranilate synthase	ICR 1	*trp E1*
			ICR 6	*trp E2*
			ICR 14	*trp E3*
			ICR 16	*trp E4*
			UV 17	*trp E5*
			SB 194	*trp E194*
2	*trp D*	PR-transferase	NA 1	*trp D1*
		InGP synthase	NA 3	*trp D2*
			NA 8	*trp D3***
			NG 61	*trp D4*
			NG 62	*trp D5*
			NG 120	*trp D6*
			ICR 10	*trp D7*
			ICR 11	*trp D8*
			ICR 19	*trp D9*
			ICR 22	*trp D10*
3	*trp F*	PRA-isomerase	NA 2	*trp F1***
		InGP synthase	NA 4	*trp F2*
			NA 6	*trp F3*
			NA 7	*trp F4*
			NA 10	*trp F5*
			NA 11	*trp F6*
			HA 1	*trp F7*
			HA 2	*trp F8***
			PH 1	*trp F9***
			ICR 12	*trp F10*
			SB 11	*trp F11*
			NA 5	*trp F12***
4	*trp C*	InGP synthase	NG 1	*trp C1*
			NG 2	*trp C2*
			NG 3	*trp C3*
			NG 5	*trp C4*
			NG 24	*trp C5*
			UV 15	*trp C6*
			UV 16	*trp C7*
			UV 18	*trp C8*
			EMS 1	*trp C9*
			EMS 2	*trp C10*
			168	*trp C168*
5a	*trp A*	Tryptophan synthase A	NG 63	*trp A1*
			NA 9	*trp A2*
			T 50	*trp A3***
			T 51	*trp A4***
			ICR 7	*trp A5*
			ICR 17	*trp A6*
			ICR 21	*trp A7*
5b	*trp B*	Tryptophan synthase B	NG 4	*trp B1*
			NG 6	*trp B2*

TABLE 1—Continued

Mutant class		Enzymatic defect	Mutant number	
Phenotypic*	Genotypic		Old designation†	New designation
			NG 7	*trp B3*
			NG 14	*trp B4*
			NG 23	*trp B5*
			NG 57	*trp B6*
			NG 58	*trp B7*
			NG 59	*trp B8*
			NG 60	*trp B9*
			ICR 8	*trp B10*
			ICR 9	*trp B11*
			ICR 13	*trp B12*
			ICR 15	*trp B13*
			ICR 18	*trp B14*
			ICR 20	*trp B15*

* The accumulation properties, growth responses, and enzymatic defects of these mutants have been described previously (WHITT and CARLTON 1968).

** Double mutants carrying the *E194* marker.

† Many of these strains have been described previously by CARLTON (1966, 1967), and WHITT and CARLTON (1968).

MUTATIONAL SITE ORDERING

Class trp E *mutants*: It was possible to determine the orders of mutant sites in the class *trp E* strains with no ambiguities (Table 3a). There were, however, two regions in each of which two mutants appeared to map at the same location. As is evident from crosses 2 and 6 no recombination was obtained between mutants *E2* and *E194*, which are known to be independent mutations because they were obtained from different sources. As indicated in crosses 3 and 4, *trp E3* and *trp E4* appear also to be located at or near the same site. Although these two strains were induced in the same mutagenic experiment, they are considered to be independent

TABLE 2

Distribution of tryptophan mutants obtained by mutagenesis with different agents

	Number of mutants induced by								
Mutant class	UV†	NA*	HA*	EMS*	NG†	PH*	ICR-170†	ICR-191†	Total
1	1	.	.	.	.	.	1	3	5
2	.	3	.	.	3	.	.	4	10
3	.	7	2	.	.	1	.	1	11
4	3	.	.	2	5	.	.	.	10
5a	1	1	.	.	2	.	.	3	7
5b	.	.	.	.	9	.	.	6	15
TOTALS	5	11	2	2	19	1	1	17	58

* Obtained by direct mutagenic treatment of DNA.

† Obtained by treatment of intact cells.

TABLE 3a

Mutational-site ordering within the tryptophan gene cluster of Bacillus subtilis*

Cross	Class *trp E* mutants
(1)	*trp E1* [(12.1) (13.0) (9.7) (20.5) (25.4) / *trp E2, trp E3, trp E4, trp E5, trp E194*]
(2)	[(43.0) (48.5) / *trp E1, trp E5*] *trp E2*† (*trp E194*) [(17.1) (11.4) / *trp E3, trp E4*]
(3)	[(63.4) (41.2) (63.3) (72.6) / *trp E1, trp E2, trp E5, trp E194*] *trp E3* (*trp E4*)
(4)	[(58.7) (38.1) (63.1) (59.5) / *trp E1, trp E2, trp E5, trp E194*] *trp E4* (*trp E3*)
(5)	[(75.9) / *trp E1*] *trp E5* [(24.3) (17.2) (17.9) (10.9) / *trp E2, trp E3, trp E4, trp E194*]
(6)	[(69.7) (48.9) / *trp E1, trp E5*] *trp E194* (*trp E2*) [(22.2) (25.3) / *trp E3, trp E4*]

Deduced order: —*trp E1*—*trp E5*— [*trp E194* / *trp E2*] — [*trp E3* / *trp-E4*]

* The numbers in parentheses represent the frequency of prototrophic recombinants relative to the number of recombinants between the two sites being ordered.

† Strains included in parentheses under the recipient strain failed to recombine with the recipient.

TABLE 3b

Cross	Class *trp D* mutants
(1)	[(16.6) (18.2) / *trp E1, trp D6*] *trp D1* (*trp D2*) [(100.0) (79.3) (88.2) (85.5) (100.0) / *trp D4, trp D7, trp D8, trp D10, trp F3*]
(2)	[(24.7) (3.0) / *trp E1, trp D6*] *trp D2* (*trp D1*) [(81.1) (72.1) (98.5) (84.2) (91.7) (96.0) (88.3) (100.0) / *trp D4, trp D7, trp D8, trp D10, trp C1, trp F3, trp B7, trp A2*]
(3)	[(31.7) / *trp E1*] *trp D3* [(83.1) (76.4) (76.4) (80.1) (89.0) (85.4) (100.0) (97.4) (86.9) (80.9) (92.7) / *trpD1, trpD2, trpD4, trpD6, trpD7, trpD8, trpD10, trpC1, trpF3, trpB7, trpA2*]
(4)	[(15.6) (6.7) (2.9) (17.1) (9.0) (13.4) / *trpE1, trpD1, trpD2, trpD6, trpD7, trpD8*] *trpD4* [(47.0) (95.5) (79.2) (75.6) (81.1) / *trpD10, trpC1, trpF3, trpB5, trpA2*]
(5)	[(35.6) (31.2) (22.1) (20.6) / *trpE1, trpD1, trpD2, trpD6*] *trpD7* [(65.5) (62.9) (82.4) (67.2) (75.5) (99.9) (77.3) / *trpD4, trpD8, trpD10, trpC168, trpF2, trpB7, trpA2*]
(6)	[(0.35) (24.5) (30.9) (13.8) (22.2) / *trpE5, trpD1, trpD2, trpD6, trpD7*] *trpD8* [(80.6) (89.4) (96.4) (88.0) / *trpD4, trpD10, trpB7, trpA2*]
(7)	[(16.2) (20.2) (23.3) (34.1) (11.6) (21.2) (35.6) / *trpE1, trpD1, trpD2, trpD4, trpD6, trpD7, trpD8*] *trpD10* [(78.0) (87.1) (93.4) / *trpC6, trpF2, trpA2*]

Deduced order: —*trp D3*—*trp D6*—[*trp D1* / *trp D2*]—*trpD7*—*trp D8*—*trp D4*—*trp D10*

TABLE 3c

Cross	Class *trp C* mutants
(1)	[(0.5) *trp E5*, (7.3) *trp C2*] *trp C1* [(95.4) *trp B7*, (62.2) *trp A2*] (*trp C3*, *trp C4*, *trp C5*, *trp C6*, *trp C7*, *trp C8*, *trp C9*, *trp C10*, *trp C168*)
(2)	[(1.8) *trp E5*] *trp C2* [(47.1) *trp C1*, (51.7) *trp C3*, (59.5) *trp C4*, (55.4) *trp C5*, (62.6) *trp C6*, (57.1) *trp C7*, (65.9) *trp C8*, (59.8) *trp C9*, (52.8) *trp C10*, (56.9) *trp C168*, (100.0) *trp B7*, (62.9) *trp A2*]
(3)	[(1.0) *trp E5*, (9.1) *trp D4*, (21.8) *trp C2*] *trp C3* [(72.4) *trp B5*, (68.2) *trp A1*] (*trp C1*, *trp C4*, *trp C5*, *trp C6*, *trp C7*, *trp C8*, *trp C9*, *trp C10*, *trp C168*)
(4)	[(15.5) *trp D4*, (23.4) *trp C2*] *trp C4* [(85.6) *trp B5*, (100.0) *trp A1*] (*trp C1*, *trp C3*, *trp C5*, *trp C6*, *trp C7*, *trp C8*, *trp C9*, *trp C10*, *trp C168*)
(5)	[(0.1) *trp E5*, (14.3) *trp D4*, (20.6) *trp C2*] *trp C5* [(81.5) *trp B5*, (72.7) *trp A1*] (*trp C1*, *trp C3*, *trp C4*, *trp C6*, *trp C7*, *trp C8*, *trp C9*, *trp C10*, *trp C168*)
(6)	[(29.6) *trp E1*, (22.8) *trp C2*] *trp C6* [(98.0) *trp B5*, (83.9) *trp A1*] (*trp C1*, *trp C3*, *trp C4*, *trp C5*, *trp C7*, *trp C8*, *trp C9*, *trp C10*, *trp C168*)
(7)	[(29.4) *trp C2*] *trp C7* (*trp C1*, *trp C3*, *trp C4*, *trp C5*, *trp C6*, *trp C8*, *trp C9*, *trp C10*, *trp C168*)

(8) [(17.1) *trp C2*] *trp C8*
(*trp C1*, *trp C3*, *trp C4*, *trp C5*, *trp C6*, *trp C7*, *trp C9*, *trp C10*, *trp C168*)

(9) [(1.1) *trp E5*, (13.2) *trp C2*] *trp C9* [(73.6) *trp B5*]
(*trp C1*, *trp C3*, *trp C4*, *trp C5*, *trp C6*, *trp C7*, *trp C8*, *trp C10*, *trp C168*)

(10) [(16.4) *trp D4*, (13.8) *trp C2*] *trp C10* [(73.7) *trp B5*, (63.2) *trp A1*]
(*trp C1*, *trp C3*, *trp C4*, *trp C5*, *trp C6*, *trp C7*, *trp C8*, *trp C9*, *trp C168*)

(11) [(1.1) *trp E5*, (34.5) *trp C2*] *trp C168* [(86.7) *trp B5*, (57.8) *trp A1*]
(*trp C1*, *trp C3*, *trp C4*, *trp C5*, *trp C6*, *trp C7*, *trp C8*, *trp C9*, *trp C168*)

Deduced order: —*trp C2*— [*trp C1*, *trp C3*, *trp C4*, *trp C5*, *trp C6*, *trp C7*, *trp C8*, *trp C9*, *trp C10*, *trp C168*]

mutants since they were isolated from separate tubes in the mutation induction procedure.

Class trp D *mutants*: In this class, it was possible to establish an unambiguous map order for all the mutants with the exception of *trp D1* and *trp D2*, between which there is no apparent recombination (Table 3b). These should, however, be distinct mutants due to the fact that they were both induced by nitrous acid treatment of transforming DNA, and should, therefore, have arisen from two independent mutational events.

Mutant *trp D6* was not mapped as a recipient in these experiments because it was not possible to isolate a double mutant carrying the *trp E194* site, probably because the *trp D6* site is so close to the *trp E* region. However, it was placed to the right of *trp D3* on the basis of cross 3 in which *trp D3* was used as the recipient. Mutant *trp D3* was not mapped as a donor because it was isolated initially as a double mutant in the *trp E194* background.

TABLE 3d

Cross	Class *trp F* mutants
(1)	[(12.9) (22.6) (16.7) (21.0) (15.9) (21.1) (21.0) (18.7) *trp E1, trp D1, trp C168, trp F3, trp F4, trp F6, trp F7, trp F10*] *trp F1* [(61.3) (88.8) *trp B7, trp A2*] (*trp F2, trp F5*)
(2)	[(32.1) (5.0) (12.3) (16.7) (6.4) (15.2) (32.7) (25.6) *trp E1, trp D1, trp C168, trp F3, trp F4, trp F6, trp F7, trp F10*] *trp F2* [(68.3) (62.2) *trp B7, trp A2*] (*trp F5*)
(3)	[(8.5) (3.7) (4.5) (18.4) *trp E1, trp D1, trp C168, trp F6*] *trp F3* [(67.5) (56.0) (55.6) (39.8) (64.2) (55.5) *trp F2, trp F5, trp F7, trp F10, trp B7, trp A2*] (*trp F4*)
(4)	[(6.9) (7.3) (10.6) (25.8) *trp E1, trp D1, trp C168, trp F6*] *trp F4* [(53.0) (56.1) (50.0) (69.5) (88.3) *trp F2, trp F5, trp F10, trp B7, trp A2*] (*trp F3*)
(5)	[(20.6) (0.4) (5.8) (18.0) (8.6) (17.3) (24.7) (17.7) *trp E1, trp D1, trp C168, trp F3, trp F4, trp F6, trp F7, trp F10*] *trp F5* [(68.8) (66.0) *trp B7, trp A2*] (*trp F2*)
(6)	[(13.5) (14.0) (20.3) *trpD1, trpC168, trpF6*] *trpF8* [(78.2) (42.8) (56.8) (53.7) (73.0) (70.6) (77.2) (79.6) *trpF2, trpF3, trpF4, trpF5, trpF7, trpF10, trpB7, trpA2*]
(7)	[(6.2) (7.7) (6.5) (14.9) (16.8) (12.4) (15.9) *trp E1, trp D1, trp C168, trp F3, trp F4, trp F7, trp F10*] *trp F9* [(60.2) (72.4) *trp B7, trp A2*] (*trp F2, trp F5*)
(8)	[(7.8) (5.9) (10.5) (30.9) (33.7) (16.8) *trpE1, trpD4, trpC6, trpF3, trpF4, trpF6*] *trpF10* [(62.2) (38.7) (65.6) (74.1) (84.1) *trpF2, trpF5, trpF7, trpB7, trpA2*]
(9)	[(8.5) (5.9) (4.5) (10.1) *tryE1, trpD1, trpC168, trpF6*] *trpF12* [(46.1) (59.1) (51.2) (51.8) (62.7) (76.7) *trpF2, trpF5, trpF7, trpF10, trpB7, trpA2*] (*trp F3, trp F4*)
Deduced order:	—*trp F6*—*trp F8*—[*trp F3, trp F4,* / *trp F12*]—*trp F10*—*trp F7*—[*trp F1, trp F2,* / *trp F5, trp F9*]

Mutant *trp D5* was not mapped against the other *D* mutant strains because it arose in the same tube as *trp D4* in the initial mutagenesis experiment. Mutant *trp D9* was not mapped because it exhibited a very high frequency of reversion.

Class trp C *mutants*: Mapping experiments with this class of mutants established only that *trp-C2* maps to the left of the other mutations. None of the other mutants in this class was found to recombine with each other (Table 3c), although they all mapped to the right of *trp C2* and to the left of all the *trp-F* mutants. In a number of crosses, a ten-fold greater number of recipient cells treated with donor DNA was plated in order to increase the level of sensitivity, but this procedure also failed to produce any recombinants. However, the recipients were shown to be competent since they gave high levels of prototrophic recombinants with wild-type DNA.

Class trp F *mutants*: As is apparent from the crosses involving mutants in this class (Table 3d), two small clusters exist; one consists of *trp F3*, *trp F4*, and

TABLE 3e

Cross	Class *trp B* mutants
(1)	[(19.1) (8.2) (3.6) (24.6) (14.0) (14.3) (26.2) (19.7) (24.5) (32.9) *trp F2, trp C168, trp D4, trp B3, trp B4, trp B5, trp B7, trp B11, trp B13, trp B15*] *trp B10* [(57.4) (47.6) (52.8) (66.0) *trp B1, trp B8, trp B9, trp A2*] *trp B10*
(2)	[(8.1) (6.2) (26.8) *trp C168, trp D4, trp F2*] *trp B11* [(63.2) (45.7) (50.0) (65.0) (52.0) (59.2) (72.1) (76.2) (78.2) (67.8) (63.8) *trp B1, trp B4, trp B5, trp B7, trp B8, trp B9, trp B10, trp B12, trp B13, trp B15, trp A2*]
(3)	[(2.9) (4.0) (5.0) (12.0) (23.1) (21.0) (10.1) (19.9) (9.8) (22.7) (29.3) *trp E5, trp C168, trp D4, trp F2, trp B3, trp B4, trp B5, trp B7, trp B11, trp B13, trp B15*] *trp B12* (*trp B10*) [(51.1) (44.5) (49.3) (49.8) *trp B1, trp B8, trp B9, trp A2*]
(4)	[(6.7) (3.7) (29.0) (24.7) (4.0) (6.0) (24.7) (17.2) (24.0) *trp C168, trp D4, trp F2, trp B3, trp B4, trp B5, trp B7, trp B11, trp B13*] *trp B15* [(48.3) (76.0) (37.4) (63.5) (56.0) (52.2) *trp B1, trp B8, trp B9, trp B10, trp B12, trp A2*] *trp B15*
(5)	[(16.4) (31.5) (9.4) (35.1) (19.9) (22.6) *trp E1, trp B10, trp B11, trp B12, trp B13, trp B15*] *trp B1* (*trp B8*) [(45.1) *trp A7*]
(6)	[(15.6) *trp B11*] *trp B2* [(70.5) *trp B12*]
(7)	[(5.2) *trp E1*] *trp B3* [(71.8) (60.3) (67.6) (51.1) (65.7) *trp B10, trp B12, trp B13, trp B15, trp A5*]
(8)	[(13.1) *trp B11*] *trp B4* [(60.3) (58.5) *trp B10, trp B13*]
(9)	[(1.5) (1.2) (2.9) (10.2) *trp D1, trp C2, trp F2, trp B11*] *trp B5* [(72.4) (68.0) (38.5) (50.0) (50.9) *trp B7, trp B10, trp B12, trp B13, trp A2*]
(10)	[(9.5) (1.9) (1.7) (10.9) (12.5) *trp E1, trp C1, trp D4, trp F5, trp B11*] *trp B7* [(61.7) (47.1) (56.6) (74.6) (42.3) *trp B10, trp B12, trp B13, trp B15, trpA1*]
(11)	[(20.8) (21.5) (19.2) (11.4) (16.5) (23.0) *trp E1, trp B3, trp B7, trp B11, trp B13, trp B15*] *trp B8* (*trp B1*) [(50.6) (43.8) (50.9) *trp B9, trp B10, trp B12*]
(12)	[(10.2) (13.0) (19.1) (22.0) (8.2) (16.8) (14.1) (13.0) *trp D4, trp C2, trp F2, trp B10, trp B11, trp B12, trp B13, trp B15*] *trp B9* [(90.7) *trp A2*]
Deduced order:	—*trp B11*—[*trp B2* / *trp B5*]—*trp B6*—*trp B4*—*trp B7*—*trp B3*—*trp B13*—*trp B15*— —[*trp B10* / *trp B12*]—[*trp B1* / *trp B8*]—*trp B9*—

trp F12 which were not resolvable from one another, and the other consists of *trp F1*, *trp F2*, *trp F5*, and *trp F9* all of which failed to recombine.

Mutants *trp F1*, *trp F8*, *trp F9*, and *trp F12* were not used as donors in the mapping tests since they were isolated initially as double mutants carrying the

TABLE 3f

Cross	Class *trp A* mutants
(1)	[(6.7) (7.6) (14.3) (22.4) *trp E1, trp D1, trp F5, trp B10*] *trp A1* [(59.1) (38.7) (48.3) (50.8) *trp A2, trp A5, trp A6 trp A7*]
(2)	[(3.4) (9.2) (0.9) (12.7) (23.4) (16.3) (19.1) (10.5) *trp D1, trp F5, trp C168, trp B12, trp A1, trp A5, trp A6, trp A7*] *trp A2*
(3)	[(11.8) (4.6) (20.8) (16.1) (23.0) (20.0) (20.0) (24.4) *trp C1, trp D4, trp B10, trp A1, trp A2, trp A5, trp A6, trp A7*] *trp A3*
(4)	[(7.1) (3.6) (31.8) (31.3) (27.6) *trp C168, trp D4, trp B9, trp A1, trp A5*] *trp A4* [(53.0) (63.7) (48.5) *trp A2, trp A6, trp A7*]
(5)	[(9.2) (16.1) (3.7) (32.4) (24.1) *trp C168, trp F2, trp D4, trp B7, trp A1*] *trp A5* [(63.8) (86.6) (56.2) *trp A2, trp A6, trp A7*]
(6)	[(0.1) (4.5) (17.9) (5.9) (27.3) (21.2) *trp E5, trp D4, trp F2, trp C168, trp A1, trp A5*] *trp A6* [(50.0) (63.6) *trp A2, trp A7*]
(7)	[(30.9) (5.0) (20.2) (24.3) (23.9) (28.0) *trp E1, trp D4, trp B7, trp A1, trp A5, trp A6*] *trp A7* [(34.3) *trp A2*]
Deduced order:	*—trp A1—trp A5—trp A4—trp A6—trp A7—trp A2—trp A3—*

trp E194 marker. For this reason the order of these markers was based only on the basis of their behavior as recipients, Mutants *trp F6* and *trp F7* were used only as donors due to the fact that it was not possible to isolate double mutants carrying those markers and the *trp E194* reference marker. Again reciprocal crosses were not possible, but these strains were used as donors in numerous crosses and their map positions were assigned on the basis of their behavior as donors in crosses with the other strains.

Class trp B *mutants*: The orders of mutants *trp B1* through *trp B9* have been previously established (CARLTON 1966); in this study *trp B* mutants *10* through *15* were ordered in relation to those mapped earlier (Table 3e).

The only discrepancy in this region involves mutant *trp B8* when it is used as a recipient, in which case *trp B10* and *trp B12* appear to map to the right. However, when *trp B10* and *trp B12*, or *trp B1* (which cannot be resolved from *trp B8*) are used as recipients, *trp B10* and *trp B12* map to the left of *trp B8*.

Mutants *trp B13* and *trp B14* were not used as recipients in these experiments.

Class trp A *mutants*: All of the sites of *A* mutants were arranged in a unique order (Table 3f). Mutants *trp A3* and *trp A4* were used only as recipients since they were originally isolated as double mutants and carried the *trp E194* marker as well as the lesions in the *A* gene.

REVERSION STUDIES

The results obtained from the reversion studies are presented in Table 4. Since no reversion was induced in any of the strains by 2-aminopurine, 5-deoxybromouridine, hydroxylamine or nitrous acid, these mutagens are not indicated in the table.

TABLE 4

Reversion responses of tryptophan auxotrophs of B. subtilis

Strain	Mutagen used to induce	Nitrosoguanidine	ICR 170	ICR 191	Ethyl methanesulfonate	Spontaneous
trp E5	UV	+	—	—	+	—
trp F11	UV	+	—	—	+	—
trp E2	ICR 170	—	—	+	—	—
trp A5	ICR 191	—	+	+	—	—
trp B10	ICR 191	+	+	+	+	+
trp B11	ICR 191	+	—	—	—	—
trp D7	ICR 191	—	+	+	—	+
trp D8	ICR 191	—	—	+	—	—
trp F10	ICR 191	+	—	+	+	—
trp B12	ICR 191	+	+	+	+	—
trp E3	ICR 191	—	—	+	—	—
trp E4	ICR 191	—	+	+	—	—
trp A6	ICR 191	+	—	+	+	—
trp B14	ICR 191	+	+	+	+	—
trp D9	ICR 191	—	+	+	—	+
trp B15	ICR 191	—	+	+	—	—
trp A7	ICR 191	—	+	+	—	—
trp D10	ICR 191	—	+	+	—	—
trp D1	NA	+	—	—	+	+
trp D2	NA	+	—	—	+	—
trp F2	NA	—	—	—	—	+
trp F3	NA	+	—	—	+	+
trp F4	NA	+	—	—	+	+
trp F5	NA	+	—	—	+	—
trp F6	NA	+	+	+	+	—
trp C2	NG	+	—	—	+	—
trp B3	NG	+	—	—	+	+
trp B4	NG	+	—	—	+	—
trp B6	NG	+	—	—	+	—
trp B7	NG	+	—	—	+	—
trp B8	NG	+	—	—	+	—
trp B9	NG	+	—	—	+	—
trp F7	HA	+	—	—	+	+

It is evident by inspection of the data that, in all cases except one (*trp B11*), all mutants which were revertible by nitrosoguanidine were also induced to revert by EMS. Those mutants which responded to one of the ICR compounds also frequently were induced to revert by the other ICR, and in many cases also by nitrosoguanidine or EMS. Some examples are *trp B10*, *trp B12*, *trp B14*, *trp F10*, and *trp A6* all of which were reverted by EMS, nitrosoguanidine, and one or both of the ICR compounds.

Certain of the mutants, such as *trp D7*, *trp F3*, and *trp B10* exhibited rather high levels of spontaneous reversion, as evidenced by the number of prototrophic clones arising on untreated plates.

Of the 61 mutants studied, the seven double mutants and 21 of those carrying

a single marker failed to revert either spontaneously or when treated with any of the mutagens employed in these studies. These strains, which included *trp E1,194*, *trp D3,4,5,6*, *trp F1,8,9,12*, *trp C1,3,4,5,6,7,8,9,10,168*, *trp A1,2,3,4*, *trp B1,2,5*, and *13*, are eliminated from Table 4.

DISCUSSION

These studies have characterized a series of 61 tryptophan auxotrophs in regard to mutagen specificity, map position, and reversion response.

It is of interest to note that the spectrum of mutants obtained by different mutagenic treatments exhibited a certain amount of specificity. For example, nitrous acid treatment of transforming DNA yielded a majority of mutants of the class *trp F* type, while no mutants of this type were obtained by treatment of whole cells with nitrosoguanidine or ultraviolet irradiation. While these observations admittedly are based on a rather small sample of mutants, the results are considered significant. Of interest also is the high proportion of class *trp E* mutants induced by the ICR compounds, since only one other mutant was isolated in this class using other mutagens.

It is apparent from these studies that mutations representing the six phenotypic classes of tryptophan auxotrophs map in six distinct, but presumably contiguous regions that form a cluster on the genome of *B. subtilis*. It was found that all mutants in a particular class mapped in a discrete region, with no interspersing of mutants of different types (Figure 2).

There was a tendency for certain mutations in the *E*,*C*, and *F* genes to cluster, for which several possible explanations are suggested. One is that these clustered mutants represent small overlapping deletions rather than point mutations. Although many of the mutants which map in these clusters do revert, either spontaneously or by induction with certain mutagens, none of the clustered mutants in *trp C* was revertible by the mutagens employed in these studies, and thus may be of the small overlapping deletion type. A second possibility is that at least some of the strains represent repeat mutations occurring at the same site. In regard to this latter possibility, we can generally rule out that such mutants are replicas of the same initial mutational event, because routinely only one mutant was isolated from any one tube of a mutagenized cell culture. The most likely explanation for these clustered mutants is that they occur at "hot spots", or sites where mutations occur with high frequency. Evidence that certain mutants which map

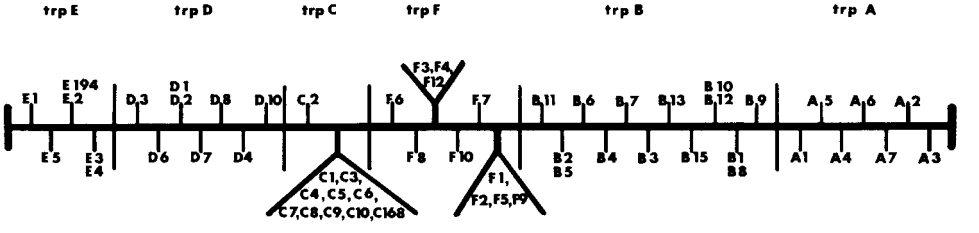

FIGURE 2.—Map of the gene cluster controlling tryptophan biosynthesis in *Bacillus subtilis*.

at the same location are actually different is provided by the reversion studies. For example, mutants *trp F3,4*, and *12* all fail to recombine; however, *trp F12* is not revertible by any of the mutagens used, while *trp F3* and *4* are reverted by nitrosoguanidine and ethyl methanesulfonate. Additional evidence leading to this conclusion is found in the differing enzyme levels of the various mutants in this cluster (WHITT and CARLTON 1968).

In the majority of the crosses made in this study, the establishment of a fine-structure order of mutants was straightforward. In one case, however, there was some ambiguity. This was found in the strain *trp B8* which mapped differently as a recipient than it did as a donor with a number of other mutants in the *trp B* region. Its map position was assigned on the basis of results with strain *trp B1* which fails to recombine with *trp B8*.

In summary, the six genes making up the tryptophan region in *B. subtilis* appear to form a contiguous section on the genome. To date, no mutants defective in the synthesis of products other than tryptophan have been found to map among the various tryptophan genes. No attempt has been made as yet in this system to carry out distance mapping using an outside reference marker, which would be necessary in order to correct for the inherent variabilities in recombination levels observed between different strains due to fluctuations in the levels of competence.

It is apparent from these studies, however, that it is feasible to carry out mutational-site ordering by a transformation procedure in order to establish a fine-structure map of the six genes in the tryptophan region. It would be of obvious value to increase the resolution of the system, possibly by using one of the recently developed techniques for separating competent cells from noncompetent cells (CAHN and FOX 1968; HADDEN and NESTER 1968; SINGH and PITALE 1968). These procedures eliminate the problem of increased background reversion levels (as occurs when a ten-fold number of cells is plated), and thus would allow us to determine whether the level of resolution permits detection of recombination between adjacent nucleotides.

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SUMMARY

Fifty-eight tryptophan auxotrophic mutants of *Bacillus subtilis* representing six phenotypic classes have been isolated following treatment of either intact cells or transforming DNA with a variety of mutagens. Mutagen specificity studies revealed that certain phenotypic classes of mutants were more readily induced by some mutagens than by others, e.g., nitrous acid treatment of transforming DNA produced mutations primarily in the *trp F* region. A marked tendency to cluster was noted for mutants of the *C* and *F* types. Reversion analyses of the various mutants using several mutagens revealed that a considerable number failed to revert. Those revertible by nitrosoguanidine also tended to be revertible by ethyl methanesulfonate, and those revertible by one of the acridine

half-mustard compounds used were frequently induced to revert by a second similar compound. None of the mutants was reverted by 2-aminopurine, 5-deoxybromouridine, hydroxylamine or nitrous acid under the conditions employed in these studies. Each of the mutants was subjected to mutational site ordering by three- point transformation crosses. With only one exception the mutations were mapped unambiguously in six distinct, but presumably contiguous, regions of the tryptophan cluster. It is concluded that the three-point transformation procedures used provide a reliable means of establishing a fine-structure map of mutational site orders for purposes of studying gene-enzyme relationships in this organism.

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