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Fine-Structure Mapping by Transformation in the Tryptophan Region of *Bacillus subtilis*

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ABSTRACT

CARLTON, BRUCE C. (Yale University, New Haven, Conn.). Fine-structure mapping by transformation in the tryptophan region of *Bacillus subtilis*. J. Bacteriol. **91**:1795–1803. 1966.—The arrangements of eight mutational sites within a segment of the tryptophan cluster of *Bacillus subtilis* have been determined by three-point reciprocal crosses with a mutation in the same gene cluster used as an outside marker. Estimates of distances between sites were made by two-point crosses and normalization to recombination values for an unlinked marker to correct for physiological variabilities between recipient strains. In general, the additivities of map distances determined by this procedure were reasonable, although some discrepancies were noted in reciprocal crosses between widely separated sites within the gene region examined. No definitive polarity effect on recombination was evident, and it appears that the variations observed in reciprocal crosses may be an effect of the probability of recovery of crossovers between exogenotic and endogenotic segments. It is concluded that fine-structure mapping is feasible for the transformation system in this organism and that a high degree of resolution can be attained.

The resolution of genes into recombinable sites has been demonstrated in a number of organisms, principally by use of sexual recombination and transduction techniques (3). Certain microorganisms (among them *Haemophilus*, *Bacillus*, *Neisseria*, pneumococcus, and several other genera), however, have not been found as yet to possess sexual reproductive systems, and in some cases no suitable temperate bacteriophages have been isolated which could be useful in transduction analysis. Each of the above groups does, however, exhibit the property of transformability by free deoxyribonucleic acid (DNA), in which a heritable transfer of genome fragments can be demonstrated [see review by Ravin (14)].

In *B. subtilis*, a gram-positive sporeforming bacterium which can be grown on completely defined medium, genetic analysis has been conducted with a wide variety of nutritional markers, and the transfer of up to 20 genes has been demonstrated by single transforming DNA particles (12). In addition, it has been possible to extend the genetic map in both directions from this gene cluster through the use of cell synchronization (17) and isolation of unfragmented DNA molecules (8), thus apparently overcoming the inherent limitations of transformation mapping due to DNA fragmentations.

B. subtilis has a number of properties which make it an ideal choice for studies of the effects of mutation on the overall physiology of the cell. In addition to its growth on a defined medium, the organism produces a variety of extracellular enzymes, including proteases and nucleases, and such diverse materials as pigments, flagella, mesosomes, antibiotics, and bacteriocins. Ultimately, it would be desirable to study the effects of genetic alteration on the structure and biological activity of a wide variety of such cellular materials, particularly in view of the possibilities for in vitro mutation of transforming DNA (10) for the isolation of new mutant types produced by specific mutagens.

Although it has been shown that the linkage of markers can be preserved during transfer by either transformation or by transduction (5), and that estimates of map distances between markers can be obtained by transformation (2, 6, 9), no conclusive study of fine-structure genetic analysis by transformation has yet been reported in *B. subtilis*. To date, only one extensive study of transformation fine-structure analysis has been reported, that of the ami A locus in pneumococcus (15).

The investigations reported here were initiated with the objectives of assessing the feasibility of the transformation technique for determining

marker orders within a single gene of *B. subtilis*, and the relative effectiveness of three-point versus two-point transformation mapping relative to the same techniques employed in other microbial systems.

MATERIALS AND METHODS

The *B. subtilis* tryptophan-requiring strains used here were derived from a prototrophic derivative of the original *tryp*$^-$ strain (168) of Spizizen (16) by nitrosoguanidine treatment. B-type mutants were characterized by their absolute requirements for tryptophan, by the accumulation of indole in culture filtrates, and by the lack of tryptophan synthetase activity in whole cells and extracts (18). Other tryptophan mutants were characterized by their growth responses to tryptophan, indole, or anthranilic acid, and by the accumulation of anthranilic acid, the Amadori intermediate, or indole glycerol (1, 4).

For three-point genetic crosses, double mutants with the *anth*$^-$ marker [strain SB 194, Nester et al. (12)] were constructed by transforming a *his*$_2^-$ *anth* $\overline{194}$ recipient with DNA prepared from strains carrying the desired *tryp-B* markers and selecting for *his*$^+$ transformants, of which about 50% had incorporated the *tryp*$^-$ marker from the donor DNA. The suspected *anth*$^-$ *tryp*$^-$ double mutants were tested for retention of the *anth*$^-$ marker by their failure to accumulate indole and the failure to yield *anth*$^+$ recombinants when treated with DNA from the original *anth*$^-$ strain.

For the two-point crosses, double mutant recipients were constructed by transformation of the various *tryp-B* strains with donor DNA from a strain carrying a marker (*his*$_1^-$) unlinked to the *tryp* region. *His*$_1^-$ *tryp*$^-$ double mutants were obtained by replica-plating from doubly supplemented medium to singly supplemented media and selecting clones with the dual requirements.

The media for preparation of competent cultures were as described by Nester et al. (12). DNA was prepared by the procedure of Nester and Lederberg (11), and was added routinely at final concentrations of 0.2 μg/ml, which under these conditions was 10-fold below saturation levels. Competent recipient cells were exposed to DNA for 20 min at 30 C, and the reaction was terminated by the addition of 20 μg/ml of deoxyribonuclease (final concentration). After centrifugation, the cells were suspended in 1 ml of minimal medium, and appropriate concentrations were plated, usually in triplicate, on minimal agar and minimal agar containing 10 μg/ml of anthranilic acid. Tryptophan-independent recombinants on minimal agar were scored as the percentage of those growing on anthranilic acid-agar. In scoring in this manner, no significant deviations were noted from the more time-consuming procedure of replica-plating. Reciprocal crosses were carried out in all cases, and all of the crosses were performed in duplicate on different days.

The procedures for two-point crosses were similar except that plating was on singly supplemented histidine- or tryptophan-agar (20 μg/ml, final concentration). Tryptophan- or histidine-independent recombinants were scored as the fraction of the total recipient cell population. Replica-plating tests confirmed that less than 1% of the recombinants selected on the singly supplemented histidine or tryptophan plates had also obtained the other marker.

RESULTS

In these experiments, the rationale for the determination of marker order within the *tryp-B* locus was as shown in Fig. 1. In configuration A, when the incoming (donor) *tryp-B* marker is to be located between the *anth*$^-$ and *tryp-B*$^-$ markers in the recipient, the frequency of *anth*$^+$ recombinants relative to *anth*$^-$ recombinants should be much lower than if the incoming marker is located to the right of the *tryp-B* recipient site, owing to the necessity of the extra double crossover event in the former case. In the B configuration, the frequency of *anth*$^+$ recombinations should be high in either case, since one double crossover event would yield *anth*$^+$ types whether the incoming *tryp-B* marker is to the left or to the right of the *tryp-B* marker in the recipient.

The results of the reciprocal three-point crosses with nine *tryp-B* mutants are presented in Table 1. It is clear from the frequencies of *anth*$^+$ recombinants in the A configuration (*anth*$^-$ marker in the recipient) that two major classes of recombination frequencies are obtained, one which is in the range of 5 to 30% of the *anth*$^-$ class, and one which is in the 40 to 60% range. In the

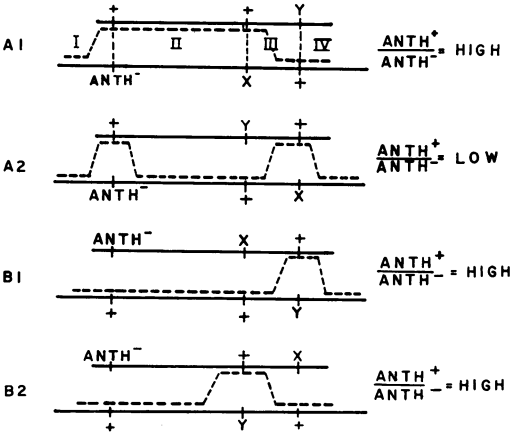

FIG. 1. *Diagrammatic representation of the rationale for mutational site ordering of tryptophan B mutants. In configuration A, if the mutant site in the donor is to the right of the site in the recipient, a high level of anth*$^+$ *relative to B*$^+$ *recombinants is expected (A1), whereas if the donor site is to the left of the recipient site a low level of anth*$^+$ *recombinants is expected relative to the B*$^+$ *class (A2). In the B configuration (doubly mutant donor), high levels of anth*$^+$ *types are to be expected in either case.*

B configuration, most of the *anth*$^+$ frequencies are in excess of 70%, and in many instances approach 100% of the B$^+$ class. The data are summarized in Table 2 and Fig. 2, in which the relative orders of any two markers are assigned on the basis of whether the A configuration yields low (up to 30%) or high (over 30%) levels of *anth*$^+$ recombinants relative to B$^+$ types. As is evident from these data, eight of the nine B mutants can be placed within the tryptophan region in an unambiguous order. The two mutants B6 and B23 cannot be ordered in these experiments, since no significant level of recombination takes place between the two markers. Although it cannot be ruled out in the absence of protein-structure information that these represent very closely linked mutations, the more probable explanation is that they are mutations at the same site.

An approximate indication of the degree of resolution by transformation fine-structure mapping can be obtained from the data in Table 3. The frequency of recombination between the two most distal B mutant sites tested, B60 and B23, is approximately 25 times that of the closely linked sites B58 and B7 or B59 and B7. These values can be contrasted to transformation levels for an unlinked marker (*his*$_1^-$), which under the conditions employed in these experiments fall within the range of 10^{-3} to 3×10^{-2} relative to the recipient cell input.

The results of a series of reciprocal two-point recombination crosses between several pairs of mutants is presented in Table 4. It is evident from the data that there are extreme variations in the competence levels of the strains involved, as measured by the transformation levels for the unlinked histidine marker. These variations are also reflected in the levels of *tryp*$^+$ recombinants obtained, which taken by themselves are essentially unrelated to the orders of the markers as ascertained by the three-point tests. However, when the *tryp*$^+$ recombination values are normalized to the transformation values for the unlinked histidine marker, the map distances assume more meaningful values. As shown in Fig. 3, the additivities are, while not absolute, reasonable for this limited series of crosses within the *tryp-B* region. One further point bears comment with regard to the data in Table 4, namely, the variations observed for recombination values in reciprocal crosses. While, for most of the crosses, the *tryp*$^+$ recombination frequencies are greatest when the donor marker is to the right of the *tryp-B* marker in the recipient (crosses 1, 3, 7, 16, 17, and 21), there are cases when the recombination frequency is greater when the donor marker is to the left of the recipient marker (crosses 5 and 9). When these values are normalized to the *his*$^+$ transformation frequency, in many instances these differences are removed, and the corrected recombination values for reciprocal crosses are in remarkably good agreement (reciprocal crosses 1 and 2, 9 and 10, 11 and 12, 15 and 16, 17 and 18). It would thus appear that the process of normalizing absolute recombination frequencies between mutant sites to that for an unlinked marker leads to reasonable estimates of map distances in the *B. subtilis* system.

DISCUSSION

These experiments have demonstrated that mutational sites within a single gene can be ordered by three-point transformation crosses, using as an outside marker a mutation within the same gene cluster. The three-point crossing procedures utilized in the experiments reported here have the decided advantages of (i) minimal interference due to competence variabilities between different recipient strains since all recombination values are scored as the ratio of recombinants in one region to those in a closely linked region; and (ii) they apparently overcome any difficulties due to differential marker efficiencies between mutants (7). That competence variations are a definite factor in the *B. subtilis* system is shown by the widely different levels for incorporation of the histidine locus in the two-point crosses (Table 4). This variable is essentially eliminated in the three-point crosses where the outside marker is so close as to be carried on the same transforming DNA molecule (12), so that by scoring the ratios of recombinants in one region relative to those in a closely associated region, the competence problem is automatically corrected for. With regard to the marker efficiency problem, the explanation as to why this problem is overcome is not so obvious. Although in these experiments the marker efficiencies have not been directly determined in one-point crosses with wild-type donor DNA, the results of the two-point experiments would appear to indicate some degree of marker efficiency differences in the mutants studied. One possibility that may be considered is that each series of three-point crosses involving the same double mutant recipient with a series of single mutant donor DNA molecules automatically corrects for the effects of marker efficiencies and physiological variations in the recipient strains so as to give accurate marker orders. A second possible explanation is that each three-factor cross can be visualized as the sum of two two-point crosses, which, since they are localized

TABLE 1. *Recombination data for reciprocal crosses of tryp B mutants*

Recipient	Donor	Per cent *anth*+	Proposed marker order
anth B4	anth	0	
anth	anth B4	0	—
anth B4	PRT 3	6.0	
PRT 3	anth B4	100	anth-PRT 3-B4
anth B4	A63	45.8	
A63	anth B4	78.5	anth-B4-A63
anth B4	B4	0	
B4	anth B4	0	—
anth B4	B6	12.0	
B6	anth B4	90.4	anth-B6-B4
anth B4	B7	17.4	
B7	anth B4	53.0	anth-B7-B4
anth B4	B14	21.9	
B14	anth B4	62.4	anth-B14-B4
anth B4	B23	16.8	
B23	anth B4	100	anth-B23-B4
anth B4	B57	13.5	
B57	anth B4	82.9	anth-B57-B4
anth B4	B58	20.8	
B58	anth B4	97.9	anth-B58-B4
anth B4	B59	28.6	
B59	anth B4	94.6	anth-B59-B4
anth B4	B60	64.1	
B60	anth B4	90.3	anth-B4-B60
anth B6	anth	0	
anth	anth B6	0	—
anth B6	PRT 3	5.2	
PRT 3	anth B6	100	anth-PRT 3-B6
anth B6	B4	86.4	
B4	anth B6	67.8	anth-B6-B4
anth B6	B6	0	
B6	anth B6	0	—
anth B6	B7	50.9	
B7	anth B6	71.9	anth-B6-B7
anth B6	B14	57.8	
B14	anth B6	98.4	anth-B6-B14
anth B6	B23	0	
B23	anth B6	0	anth- $\frac{B6}{B23}$
anth B6	B57	52.7	
B57	anth B6	87.5	anth-B6-B57
anth B6	B58	60.6	
B58	anth B6	72.2	anth-B6-B58
anth B6	B59	70.6	
B59	anth B6	87.4	anth-B6-B59
anth B6	B60	85.7	
B60	anth B6	91.0	anth-B6-B60
anth B7	anth	0	
anth	anth B7	0.02	—
anth B7	PRT 3	4.7	
PRT 3	anth B7	100	anth-PRT 3-B7
anth B7	A63	64.1	
A63	anth B7	78.2	anth-B7-A63
anth B7	B4	50.6	
B4	anth B7	96.1	anth-B7-B4
anth B7	B6	18.8	
B6	anth B7	91.1	anth-B6-B7
anth B7	B7	0	
B7	anth B7	0	—
anth B7	B14	29.0	
B14	anth B7	73.6	anth-B14-B7
anth B7	B23	20.8	
B23	anth B7	100	anth-B23-B7
anth B7	B57	11.2	
B57	anth B7	56.4	anth-B57-B7
anth B7	B58	30.6	
B58	anth B7	73.4	anth-B58-B7
anth B7	B59	65.6	
B59	anth B7	74.9	anth-B7-B59
anth B7	B60	73.1	
B60	anth B7	76.7	anth-B7-B60
anth B14	anth	0	
anth	anth B14	0	—
anth B14	PRT 3	1.1	
PRT 3	anth B14	76.4	anth-PRT 3-B14
anth B14	A63	55.6	
A63	anth B14	100	anth-B14-A63
anth B14	B4	48.0	
B4	anth B14	88.4	anth-B14-B4
anth B14	B6	14.3	
B6	anth B14	95.3	anth-B6-B14
anth B14	B7	53.3	
B7	anth B14	82.9	anth-B14-B7
anth B14	B14	0	
B14	anth B14	0	—
anth B14	B23	19.6	
B23	anth B14	94.7	anth-B23-B14
anth B14	B57	21.4	
B57	anth B14	88.3	anth-B57-B14
anth B14	B58	49.4	
B58	anth B14	85.0	anth-B14-B58
anth B14	B59	55.2	
B59	anth B14	83.3	anth-B14-B59
anth B14	B60	71.8	
B60	anth B14	85.8	anth-B14-B60
anth B23	anth	0	
anth	anth B23	0	—
anth B23	PRT 3	11.1	
PRT 3	anth B23	88.7	anth-PRT 3-B23
anth B23	A63	87.8	
A63	anth B23	98.0	anth-B23-A63
anth B23	B4	64.9	
B4	anth B23	87.5	anth-B23-B4

TABLE 1.—*Continued*

Recipient	Donor	Per cent *anth*+	Proposed marker order
anth B23	B6	0	
B6	anth B23	0	anth-$\frac{B23}{B6}$
anth B23	B7	70.1	
B7	anth B23	98.2	anth-B23-B7
anth B23	B14	54.6	
B14	anth B23	87.3	anth-B23-B14
anth B23	B23	0	
B23	anth B23	0	—
anth B23	B57	42.5	
B57	anth B23	42.0	anth-B23-B57
anth B23	B58	59.3	
B58	anth B23	100	anth-B23-B58
anth B23	B59	66.4	
B59	anth B23	50.1	anth-B23-B59
anth B23	B60	64.8	
B60	anth B23	83.4	anth-B23-B60
anth B57	anth	0	
anth	anth B57	0	—
anth B57	PRT 3	1.2	
PRT 3	anth B57	100	anth-PRT 3-B57
anth B57	B4	73.8	
B4	anth B57	78.8	anth-B57-B4
anth B57	B6	19.4	
B6	anth B57	94.0	anth-B6-B57
anth B57	B7	50.4	
B7	anth B57	90.1	anth-B57-B7
anth B57	B14	70.9	
B14	anth B57	96.5	anth-B57-B14
anth B57	B23	28.8	
B23	anth B57	95.0	anth-B23-B57
anth B57	B57	0	
B57	anth B57	0	—
anth B57	B58	61.3	
B58	anth B57	70.0	anth-B57-B58
anth B57	B59	59.2	
B59	anth B57	94.9	anth-B57-B59
anth B57	B60	70.1	
B60	anth B57	95.4	anth-B57-B60
anth B58	anth	0	
anth	anth B58	0	—
anth B58	PRT 3	6.6	
PRT 3	anth B58	94.6	anth-PRT 3-B58
anth B58	A63	48.3	
A63	anth B58	91.5	anth-B58-A63
anth B58	B4	46.7	
B4	anth B58	100	anth-B58-B4
anth B58	B6	13.7	
B6	anth B58	96.8	anth-B6-B58
anth B58	B7	41.4	
B7	anth B58	69.7	anth-B58-B7
anth B58	B14	28.6	
B14	anth B58	51.4	anth-B14-B58

TABLE 1.—*Continued*

Recipient	Donor	Per cent *anth*+	Proposed marker order
anth B58	B23	14.6	
B23	anth B58	100	anth-B23-B58
anth B58	B57	13.0	
B57	anth B58	66.6	anth-B57-B58
anth B58	B59	61.4	
B59	anth B58	92.0	anth-B58-B59
anth B58	B60	40.6	
B60	anth B58	100	anth-B58-B60
anth B59	anth	0	
anth	anth B59	.0001	—
anth B59	PRT 3	6.3	
PRT 3	anth B59	97.8	anth-PRT 3-B59
anth B59	A63	63.1	
A63	anth B59	—	anth-B59-A63
anth B59	B4	50.5	
B4	anth B59	85.5	anth-B59-B4
anth B59	B6	11.0	
B6	anth B59	100	anth-B6-B59
anth B59	B7	25.6	
B7	anth B59	43.8	anth-B7-B59
anth B59	B14	20.9	
B14	anth B59	65.2	anth-B14-B59
anth B59	B23	13.8	
B23	anth B59	100	anth-B23-B59
anth B59	B57	12.5	
B57	anth B59	33.1	anth-B57-B59
anth B59	B58	18.8	
B58	anth B59	100	anth-B58-B59
anth B59	B59	0	
B59	anth B59	0	—
anth B59	B60	50.9	
B50	anth B59	91.0	anth-B59-B60
anth B60	anth	0	
anth	anth B60	0	—
anth B60	PRT 3	5.2	
PRT 3	anth B60	99.3	anth-PRT 3-B60
anth B60	B4	22.9	
B4	anth B60	34.5	anth-B4-B60
anth B60	B6	6.5	
B6	anth B60	87.9	anth-B6-B60
anth B60	B7	6.5	
B7	anth B60	36.6	anth-B7-B60
anth B60	B14	10.7	
B14	anth B60	35.0	anth-B14-B60
anth B60	B23	9.7	
B23	anth B60	89.5	anth-B23-B60
anth B60	B57	8.2	
B57	anth B60	71.1	anth-B57-B60
anth B60	B58	8.8	
B58	anth B60	54.4	anth-B58-B60
anth B60	B59	25.1	
B59	anth B60	91.0	anth-B59-B60
anth B60	B60	0	
B60	anth B60	0	—

TABLE 2. *Summary of recombination data for three-point transformation crosses**

Anth⁻*B*⁻ recipient	*B*⁻donor										Relative marker orders
	4	6	7	14	23	57	58	59	60	3†	
4	0	12.0	17.4	21.9	16.8	13.5	20.8	28.6	64.1	6.0	[3] [6, 7, 14, 23, 57, 58, 59] [4] [60]
6	86.4	0	50.9	57.8	0	52.7	60.6	70.6	85.7	5.2	[3] [6, 23] [4, 7, 14, 57, 58, 59, 60]
7	50.6	18.8	0	29.0	20.8	11.2	30.6	65.6	73.1	4.7	[3] [6, 14, 23, 57, 58] [7] [4, 59, 60]
14	48.0	14.3	53.3	0	19.6	21.4	49.4	55.2	71.8	1.1	[3] [6, 23, 57] [14] [4, 7, 58, 59, 60]
23	64.9	0	70.1	54.6	0	42.5	59.3	66.4	64.8	11.1	[3] [6, 23] [4, 7, 14, 57, 58, 59, 60]
57	73.8	19.4	50.4	70.9	28.8	0	61.3	59.2	70.1	1.2	[3] [6, 23] [57] [4, 7, 14, 58, 59, 60]
58	46.7	13.7	41.4	28.6	14.6	13.0	0	61.4	40.6	6.6	[3] [6, 14, 23, 57] [58] [4, 7, 59, 60]
59	50.5	11.0	25.6	20.9	13.8	12.5	18.8	0	50.9	6.3	[3] [6, 7, 14, 23, 57, 58] [59] [4, 60]
60	22.9	6.5	6.5	10.7	9.7	8.2	8.8	25.1	0	5.2	[3] [4, 6, 7, 14, 23, 57, 58, 59] [60]

* Expressed as *anth*⁺ percentage in *B*⁺ recombinant class.
† PRT 3 reference marker.

within a very short region of the genome, automatically correct for variations in the recombination process normally observed between unlinked regions of the genome. Although neither of these possibilities sheds any light on the underlying causes for marker efficiency variations, they are offered as possible explanations as to why these effects are minimized by the use of three-factor crosses.

In considering the results of two-point crosses and relative map distances, it would appear as if the procedure of normalizing recombination values between sites within the region under study to those obtained for recombination of an unlinked marker lead to reasonably additive values over the short regions examined in these experiments (Fig. 3). Whereas variations are observed between the values obtained in some reciprocal crosses, in others the agreement is quite good, particularly when the interval being measured is a short one (Table 4) Unlike the results obtained by Anagnostopoulous and Crawford (2), there does not appear to be a definitive polarity effect in the recombination values between sets of mutants; that is, the level of recombination is not always related to which mutant site is in the recipient and which is contributed by the donor. Although the two-point data presented here are not extensive, it would appear that the most likely explanation for the variations in recombination frequencies in reciprocal crosses does not reside in the *direction* in which the recombination process takes place, but rather has to do with the *mechanism* by which markers may be included or excluded by a recombination event. This effect can be examined more closely in the data for reciprocal three-factor crosses (Table 1). As diagrammed in Fig. 1, the rationale for ordering mutant sites in three-factor crosses resides in the predicted

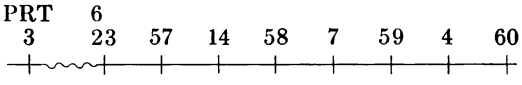

FIG. 2. *Assigned marker orders.*

differences in levels of recombination for the outside marker, depending on whether a quadruple crossover is required or whether a double crossover will suffice. Therefore, when a marker contained in a donor DNA fragment is situated to the right of the mutant marker in the recipient, the level of prototrophic recombinants for the outside marker relative to the recombinants for the markers being ordered should be high, as should also be the case when the reciprocal cross is carried out, since in each case a double crossover event can lead to completely prototrophic recombinants. In actuality, the ratio of *anth*⁺ to *anth*⁻ recombinant types was found to be invariably higher when the cross was carried out in the B configuration. This could indicate either that: (i), the frequencies of recombination events in region IV are greater than the frequencies in region I (since a crossover in region III is common to both reciprocal crosses); or (ii) that a switch to the endogenote has a greater probability of being recovered than a switch out to the exogenetic fragment. The first possibility could be attributable to either a polarity in the direction of recombination, as postulated for this same region of the chromosome by Anagnostopoulous and Crawford (2), or alternatively to a proximity of region I to an end of the transforming fragment (13). The results obtained in the experiments described here do not support the first of these interpretations since, if there were a polarity of recombination within the tryptophan region, it would be in the opposite direction to that suggested by the data of Anagnostopoulous and Crawford (2). With regard to the second possi-

TABLE 3. *Resolution between mutant sites within the tryptophan B gene of Bacillus subtilis*

Donor	Recipient	Recombination frequencies*		*Tryp*$^+$/*his*$^+$	Map distance
		Tryp$^+$	*His*$^+$		
B7	B58-*his*$_1^-$	2.55×10^{-6}	4.44×10^{-4}	5.75×10^{-3}	.006
B14	B58-*his*$_1^-$	2.68×10^{-5}	1.76×10^{-3}	1.52×10^{-2}	.015
B60	B23-*his*$_1^-$	6.90×10^{-5}	5.98×10^{-4}	1.38×10^{-1}	.138
B23	B60-*his*$_1^-$	3.32×10^{-4}	3.72×10^{-3}	8.93×10^{-2}	.089
B6	B57-*his*$_1^-$	5.20×10^{-6}	5.20×10^{-4}	1.00×10^{-2}	.010
B59	B7-*his*$_1^-$	1.46×10^{-7}	1.92×10^{-5}	7.61×10^{-3}	.008
B6	B23-*his*$_1^-$	$<10^{-8}$	4.12×10^{-7}	$<10^{-2}$	—
B60	IGP 168-*his*$_1^-$	7.90×10^{-4}	4.33×10^{-3}	1.82×10^{-1}	.182
WT	*His*$_1^-$	—	2.5×10^{-3} 3×10^{-2}	—	—

* Based on the *total* input of recipient cells.

TABLE 4. *Recombination values for reciprocal two-point crosses between tryp B mutants*

Cross	Donor	Recipient	Per cent recombinants		Map function
			Tryp$^+$	*His*$^+$	
1	B4	B57-*his*$^-$	.036	0.56	.065
2	B57	B4-*his*$^-$	.004	0.07	.057
3	B4	B58-*his*$^-$	.010	0.32	.031
4	B58	B4-*his*$^-$	.002	0.04	.050
5	B23	B60-*his*$^-$	.033	0.37	.089
6	B60	B23-*his*$^-$	.007	0.05	.14
7	B60	B14-*his*$^-$	.184	3.00	.061
8	B14	B60-*his*$^-$	.018	0.19	.096
9	B23	B14-*his*$^-$	.019	0.97	.020
10	B14	B23-*his*$^-$	.001	0.04	.025
11	B57	B58-*his*$^-$	.006	0.20	.030
12	B58	B57-*his*$^-$	.005	0.13	.038
13	B4	B7-*his*$^-$	.0003	0.0034	.089
14	B7	B4-*his*$^-$	.0001	0.0024	.069
15	B14	B4-*his*$^-$	.0008	0.011	.071
16	B4	B14-*his*$^-$	.0062	0.10	.062
17	B14	B57-*his*$^-$	.002	0.061	.033
18	B57	B14-*his*$^-$	.0008	0.031	.028
19	B58	B14-*his*$^-$	.0037	0.75	.050
20	B14	B58-*his*$^-$	.0027	0.176	.015
21	B7	B14-*his*$^-$	.0037	0.067	.055
22	B14	B7-*his*$^-$	.00003	0.0008	.031

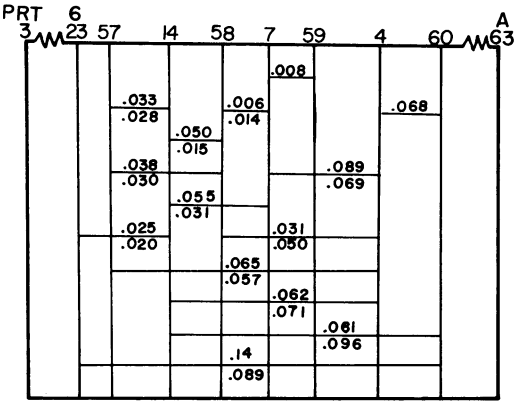

FIG. 3. *Genetic map of the tryp B mutants studied in these experiments. When two values are given for the distance between two markers, the value above the line denotes that obtained when the mutant site to the right was in the recipient; the value below the line is for the reciprocal cross when the site to the left was in the recipient. All values are expressed as the ratio of tryp recombinants to those obtained for an unlinked histidine marker (his*$_1$*).*

bility, the proximity to an end of a transforming fragment, preliminary experiments which have been carried out by zonal centrifugation of transforming DNA show no significant effect of molecular size on the ratio of *anth*$^+$ to *anth*$^-$ recombinants (B. C. Carlton, *unpublished data*). This argues against the fact that crossovers in region I are limited by the proximity to an end of a transforming fragment. The most likely explanation for the increased level of *anth*$^+$ recombinants in the B configuration appears to be that, over this small region of the genome, the probability of recovery of a switch to the endogenote is more likely than that for a switch out to the exogenote. In these particular experiments, this could be due to the fact that, in the B configuration, a switch back to the endogenote in region III is required by the selective media used. This would lead to a higher frequency of *anth*$^+$ recombinants than configuration A, where a fraction of the region III crossovers may never be recovered since they involve a switch out to the exogenote.

Finally, an estimate of the degree of resolution of mutational sites by transformation in *B. subtilis* can be made by comparison with a fine-

structure mapping system in a different organism. Yanofsky et al. (19) reported the resolution of mutational sites in the tryptophan synthetase A gene of *Escherichia coli* which are within the codon for a single amino acid of the A protein. These two sites exhibit a map distance of approximately 0.005 to 0.01, while further considerations of the colinear gene-protein relationship suggest an average figure of 0.02 map units per triplet codon of the A gene. In this transductional mapping system, the map distance is defined as the ratio of tryptophan prototrophs to transductants for an unlinked histidine marker, divided by two to correct for the differences in transduction levels for the histidine and tryptophan regions by the phage lysates.

In the *B. subtilis* mutants examined here, essentially the same procedure has been utilized for the determination of map distances, except that the values reported are uncorrected for variations in the ratio of transfer of the tryptophan-B and histidine-1 genes by transforming DNA. This has been determined to be 0.6 under the conditions used in these studies. If the assumption is made that this value represents an average ratio of replacement for any tryptophan mutant site relative to the histidine marker used in all the two-point experiments, then the observed ratios can be divided by 0.6 and the values compared with those obtained in the *E. coli* system. Of the nine mutants studied, the most distal sites (B23 and B60) have an approximate corrected map distance of 0.2 units, the more closely-linked mutants B58 and B7 (and B7 and B59) yield values of 0.017 (and 0.013). By analogy to the *E. coli* system, and in the absence of protein structure data, these values would indicate that the sites of mutational alteration in the protein would be expected to be about 10 amino acids apart for the most distal sites, and within the same or adjacent codons for the most closely linked sites. Since the structural gene for an average-sized protein of 30,000 molecular weight should reflect a total map distance of 5 to 6 units, it is obvious that the value of 0.2 observed between the most distal sites studied here is very small indeed. Whether these nine mutants actually represent a very restricted sample and are clustered in a short segment of the entire gene or whether transformation mapping leads to an amplification of the gene awaits work which can correlate the genetic data with protein structure data.

In conclusion, it appears likely that fine-structure mapping in the *B. subtilis* transformable system is, with certain limitations, as amenable to analysis as in transductional and conjugation systems, and with a degree of resolution that is equivalent to, if not greater than, the conventional mapping procedures. Whether or not inherent differences in the transformational recombination process exist relative to those operating in other genetic systems is not clear, although several possible alternatives are not favored by the results obtained in the experiments reported here.

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