Transformation Mapping of the Genes Controlling Tryptophan Biosynthesis in *Bacillus subtilis*

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Forty tryptophan auxotrophs of *Bacillus subtilis* have been placed in six phenotypic classes on the basis of growth responses, accumulation properties, and, in some cases, specific enzymatic defects. Three-point transformation crosses between representative mutants of the six different types have permitted the determination of the orders of the gene loci. In addition, mutational site orders for mutants within each of the classes have been determined by the same techniques. The organization of the cluster of genes controlling tryptophan biosynthesis in *B. subtilis* appears to be essentially analogous to that of *Escherichia coli* and *Salmonella typhimurium*.

The genetic loci which specify the structures of the enzymes concerned with tryptophan biosynthesis are organized in tightly clustered arrays on the genomes of *Escherichia coli* (15) and *Salmonella typhimurium* (5), and the regulation of expressions of these gene clusters is characteristic of operon systems (4, 9, 10). In contrast, certain fungi, including *Neurospora crassa* (1), *Saccharomyces cerevisiae* (11), and *Aspergillus nidulans* (8, 13) possess several unlinked loci which specify these enzymes, although the essential features of the biochemical pathways appear to be identical with those found in the enteric bacteria (7).

The pathway for tryptophan biosynthesis as it has been demonstrated in *E. coli*, *S. typhimurium*, and certain fungi is shown in Fig. 1. Mutant classes having the following properties have been isolated in all of these organisms: those of class B grow only on tryptophan and accumulate indole in culture filtrates; those of class A grow on either indole or tryptophan and accumulate indoleglycerol. Both of these classes are defective in the final step in tryptophan biosynthesis, the enzyme tryptophan synthetase. Mutants in class C accumulate 1-(O-carboxyphenylamino)-1-deoxyribulose-5-phosphate (CDRP), the dephosphorylated form of 1-(O-carboxyphenylamino)-1-deoxyribulose-5-phosphate (CDRP), grow on either indole or tryptophan, and are defective in the enzyme indole-3-glycerol phosphate (InGP) synthetase. Strains of the class D type accumulate an acid-labile intermediate believed to be the coupling product [N-5'-phosphoribosyl anthranilic acid (PRA)] of anthranilic acid and 5-phosphoribosyl-1-pyrophosphate (PRPP), grow on either indole or tryptophan, and are defective in the enzyme activity PRA-isomerase. A second class of fluorescence-accumulating mutants which also grow on indole or tryptophan are those of class E. These accumulate anthranilic acid in culture supernatant fluids and are defective in the enzymatic function for coupling PRPP and anthranilic acid (PR-transferase). The final class contains those mutants which respond to anthranilic acid, indole, or tryptophan, accumulate no detectable intermediate, and are defective in the first enzyme of the pathway, anthranilic acid synthetase.

The genetic and biochemical aspects of tryptophan biosynthesis in a gram-positive bacterium, *Bacillus subtilis*, have been examined for purposes of comparison with the other systems. Earlier reports of mapping studies with five tryptophan auxotrophs in four different classes placed the mutant loci in close proximity on the *B. subtilis* genome (3, 12). The present studies were aimed at the further elaboration of the genetic organization of this system and its relationship to the enzymatic steps involved.

**MATERIALS AND METHODS**

Tryptophan auxotrophs of *B. subtilis* were isolated from strain SB491, a prototrophic derivative (obtained from E. W. Nester, University of Washington) of the transformable strain 168 try^+^ (14). At the beginning of the study, mutations were induced by either nitrosoguanidine treatment (50 μg/ml in Penassay broth for 1 hr) or by ultraviolet irradiation to 10^-4^ survival. Auxotrophs were identified by plating on Nutrient Agar (Microbiological Sciences, Inc., Yonkers, N.Y.), followed by replica plating to medium lacking tryptophan, or in some cases by plating initially on tryptophan- or indole-supplemented minimal agar for the selection of tryptophan auxotrophic classes. More recently, mutants have been
induced directly in transforming deoxyribonucleic acid (DNA; prepared from wild-type strain SB 491) by treatment with nitrous acid, hydroxylamine, low pH, or ethylmethane sulfonate (EMS). These treated DNA molecules were then employed as donors to transform a recipient strain carrying a histidine requirement (his-2) which is about 50% linked to the tryptophan region (3). This technique thus allows the recovery of about half of any newly induced tryptophan auxotrophic markers in the donor DNA. It has the additional advantage that each new mutant site arising in the transforming DNA, if recovered, represents an independent mutational event. Every new mutant clone is thus assured to be unique, in contrast to the situation in which mutations are induced in dividing cells and may be duplicated during the isolation process.

New mutants were characterized as to their growth responses on anthranilic acid (10 µg/ml), indole (20 µg/ml), and L-tryptophan (20 µg/ml) in minimal agar plates. The culture supernatant fluids of 24-hr tube cultures were examined for the accumulation of indole, indoleglycerol, and fluorescent intermediates by the methods of Allen and Yanofsky (2). The activities of the various enzymes in the pathway were examined in crude extracts prepared by disruption in an Eaton pressure cell of 18-hr cultures grown in minimal salts media supplemented with 1 µg of L-tryptophan per ml, 0.5% glucose, and 0.01% casein hydrolysate (D. L. Whitt and B. C. Carlton, in preparation).

Double mutants containing the reference marker anth- (an anthranilate synthetase deficiency) were constructed by transforming an anth- his-2- recipient strain with DNA prepared from a donor strain carrying the desired additional tryptophan marker. Histidine prototrophs which carried the donor tryptophan marker were selected. The presence of the donor tryptophan marker was established by failure of the histidine prototrophs to grow on anthranilic acid-agar. The double mutant nature of these strains was verified by testing nonaccumulating recombinants (presumably due to the presence of the early anth- marker) with DNA of the anth- strain to establish that the anth- marker had remained in the recipient.

Three-point crosses for the determination of marker orders were carried out by procedures described previously (6). Recombinants were plated on minimal agar and anthranilic acid-agar, and the ratio of completely prototrophic recombinants to those carrying...
the reference anthr marker was determined. The rationale for marker order assignment was based on the expectations diagrammed in Fig. 2. For any transformation cross between a donor DNA carrying a single tryptophan mutational site and a recipient strain carrying both the reference (anthr) marker and a second tryptophan marker, it is expected that the frequency of prototrophic recombinants depends upon whether the incoming donor mutational site is located between the two mutant sites in the recipient or outside of these. As shown in the diagram, a donor site located between the two recipient sites requires a quadruple crossover event to generate the prototroph, whereas a donor site located to the right of the two sites in the recipient can yield a prototroph by a double crossover. Earlier studies of fine structure mutational site mapping of a single class of tryptophan auxotrophs had shown that the frequencies of prototrophs in such crosses fall into two classes, those yielding less than 25% and those with levels in excess of 40% (6). Marker orders were thus assigned by use of this criterion, and reciprocal crosses were carried out with the same mutant strains in both donor and recipient roles to confirm the assigned orders.

RESULTS

Mutant characterization. Table 1 presents the properties of 40 tryptophan-requiring mutants obtained by induction with the several mutagens described. Growth response and accumulation properties permitted the subdivision of these mutants into the six groups shown. Only those mutants which responded to anthranilic acid as well as indole and tryptophan were found to be nonaccumulating types, except for certain strains isolated by transformation in a recipient already carrying a block in the first enzymatic function. These mutants (class 1) have been shown to lack the enzymatic activity for the coupling of chorismic acid and L-glutamine to form anthranilic acid, whereas mutants in the other groups have this activity.

Mutants in groups 5a and 5b appear to be analogous to the A and B mutants of the tryptophan synthetase function in E. coli or S. typhimurium. The type 5a mutants grow on either indole or tryptophan, accumulate indoleglycerol (or a similar compound), and lack the ability to carry out the conversion of indoleglycerol phosphate to indole, while still possessing activity in the indole + serine → tryptophan reaction. Mutants of the 5b class lack this latter activity, grow only on tryptophan, and accumulate indole.

The remaining three mutant classes have been more difficult to resolve. Those in class 2 accumulate high levels of a fluorescent material which is chromatographically and spectrally similar to anthranilic acid. As yet it has not been possible to directly demonstrate that these mutants lack the enzymatic function expected, the PR-transferase, since it has been impossible to demonstrate this function in cell-free extracts of any strain of B. subtilis. Similarly, it is as yet not unequivocal that the class 4 mutants are those defective in the InGP synthetase function, since activity for this reaction cannot yet be found in B. subtilis. This latter class does differ from the mutant classes 2 and 3, however, by the spectral and chromatographic properties of the intermediate accumulated in culture supernatant fluids, which is less strongly fluorescent, has a slower chromatographic migration, and shows an absorption peak at 325 nm which is characteristic of 1-(O-carboxyphenylamino)-1-deoxyribulose (CDR).

Finally, the mutants of class 3, which are identical in growth and accumulation properties to those of class 2, are distinguished from the former by the absence of activity for the PR-isomerase reaction, which is found in mutants of all other classes.

Mapping studies. The results of three-point transformation crosses between representative mutants of the six different auxotrophic classes are summarized in Table 2. Although only representative crosses are shown here, all of the mutants have been similarly mapped and are found to locate in discrete regions of the tryptophan cluster. For example, in the crosses of group 2 double mutant recipients with DNA donors from each of the other groups, it is seen that all crosses yield high levels (>50%) of prototrophic recombinants for the reference anthr marker. Group 3 recipients yield low levels (<25%) of prototrophic recombinants with group 2 and group 4 donors, but high levels with group 5a and 5b donors. Strains of the group 4 type show low frequencies of anthr recombinants with group 2 mutants and high levels with all other types; those of the 5b class yield high levels with the 5a class and low levels in crosses with donors of all other types. Class 5a mutants yield low
frequencies of prototrophs in crosses with strains of all other classes.

By use of the rationale described in Fig. 2 for the ordering of markers, the various donor mutant sites can be placed either between the two markers in the recipient, in those cases where the \textit{anth} \textsuperscript{-} frequency is low, or to the right of the two recipient markers where the \textit{anth} \textsuperscript{+} frequency is high. The latter order assumes that the reference \textit{anth} \textsuperscript{+} marker is the most distal on the left according to the above orientation, which was established earlier by Anagnostopoulos and Crawford (3). By these criteria, the orders of mutational classes in this system would then be Group 1 (\textit{anth} \textsuperscript{+})—Group 2—Group 4—Group 3—Group 5b—Group 5a.

In addition to the ordering of mutational sites between different classes of mutant types, this type of analysis also permits the resolution of mutational sites between strains within the same phenotypic class. In group 2 mutants, it is seen that the mutant site in strain NG 120 is located to the left of the site in NA8, whereas both the NA1 and NG 61 sites are to the right of the NA8 site. The order of the latter two is established by the crosses with these mutant types as recipients, with the final order given as \textit{anth} \textsuperscript{+}(NG 120—NA 8—NA 1—NG 61). Similarly, within the group 4 class the order \textit{anth} \textsuperscript{+}(NG 2—168—NG 1) can be established, and within class 5a, the order \textit{anth} \textsuperscript{+}(NG 63—NA 9). Mutational site orders for mutants in the 5b class have been described previously (6). The data reported here are not sufficient to determine site orders within the group 3 class, although these have been determined by more extensive crosses (B. C. Carlton, unpublished data).

On the basis of these studies, the mutational sites affecting the various biochemical functions associated with tryptophan biosynthesis can be visualized on a genetic map as shown in Fig. 3. This representation applies to mutational site order only and is not intended to convey any implications of relative marker distances or gene sizes.

**DISCUSSION**

These studies have demonstrated the applicability of transformation techniques with the use of a classical three-point test analysis for the ordering of mutational sites over a rather extensive genetic region, in this case covering several cistrons on the bacterial chromosome. The fact that unequivocal site orders can be deduced from the frequencies of outside marker segregation in reciprocal crosses involving two sites being ordered establishes this conclusion rather firmly. This finding, in conjunction with the evidence for mutational site ordering within a single functional gene (6), places transformation mapping as a reliable and relatively simple tool for fine-structure genetic mapping in \textit{Bacillus subtilis}.
Table 2. Gene-ordering in the tryptophan cluster of Bacillus subtilis

<table>
<thead>
<tr>
<th>Y⁺ donor</th>
<th>Group 2</th>
<th>Group 4</th>
<th>Group 3</th>
<th>Group 5b</th>
<th>Group 5a</th>
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<tbody>
<tr>
<td>Anth⁻ control</td>
<td>NA1 0</td>
<td>NA8 0</td>
<td>NG61 0</td>
<td>168 0</td>
<td>NG1 0</td>
</tr>
<tr>
<td>Group 2</td>
<td>NA1 0</td>
<td>100 6</td>
<td>12 10</td>
<td>9 23 5 1</td>
<td>3 2</td>
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<tr>
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<td>0 0</td>
<td>0 0</td>
<td>0 0 0</td>
<td>0 0 0</td>
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<td>NG61</td>
<td>91 0</td>
<td>70 0</td>
<td>10 6</td>
<td>24 3 3</td>
<td>6 3</td>
</tr>
<tr>
<td>NG120</td>
<td>0 16 10</td>
<td>11 19</td>
<td>5 3</td>
<td>5 1</td>
<td>1 1</td>
</tr>
<tr>
<td>Group 4</td>
<td>168</td>
<td>95 90</td>
<td>66 0</td>
<td>68 13</td>
<td>12 6</td>
</tr>
<tr>
<td>NG1</td>
<td>84 97</td>
<td>80 60 0 55</td>
<td>14</td>
<td>14 0</td>
<td>0 0 0</td>
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<tr>
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<td>77 19 15 0</td>
<td>8 6 14</td>
<td>2 1</td>
<td>6 10</td>
</tr>
<tr>
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<td>0 0</td>
<td>0 0 0</td>
<td>0 0 0</td>
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<td>94 78</td>
<td>0 0 0</td>
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<td>92 68 100</td>
<td>0</td>
<td>0 0 0 5</td>
<td>0 0 0 9</td>
</tr>
<tr>
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<td>100 92 79</td>
<td>68 67 66</td>
<td>18 16 9</td>
<td>5 4</td>
<td>9 4</td>
</tr>
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<td>Group 5b</td>
<td>NG58</td>
<td>76 80</td>
<td>94 79</td>
<td>95 62</td>
<td>63 70</td>
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<tr>
<td>Group 5a</td>
<td>NA9</td>
<td>91 93</td>
<td>81 69 62</td>
<td>63</td>
<td>89 62</td>
</tr>
<tr>
<td>NG63</td>
<td>68 95</td>
<td>76 71</td>
<td>84 71</td>
<td>70 67</td>
<td>66</td>
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</tbody>
</table>

* Figures presented are the percentage of anth⁺ recombinants in the X⁺ Y⁺ recombinant classes.

Fig. 3. Gene map of the tryptophan cluster of Bacillus subtilis. Mutational site orders are derived from the data in Table 2, except for the group 5b and 3 mutants, which are from Carlton (6) and unpublished data.

Whether or not these conclusions will apply to the determination of map distances by these procedures remains to be determined. One facet of the work which is as yet unexplainable from these studies is the apparent tendency for clustering of mutational sites in strains defective in a given function. Whether this phenomenon is real or only apparent must be established by more extensive mapping tests with larger numbers of mutant strains induced by a variety of mutagens.

A consideration of the gene-enzyme organization of the tryptophan biosynthetic system in this gram-positive bacterium has revealed a number of points worthy of comment. Firstly, these studies have revealed two additional classes of tryptophan auxotrophs with respect to those described by other investigators (3). These are the class 3 mutants, defective in PRA-isomerase, and the class 5a mutants, which correspond to A gene mutants of the E. coli and S. typhimurium...
systems. Secondly, there is the finding that the map orders of the class 2 mutants in the present studies appear to differ from the orders established for these mutant types by the studies of Anagnostopoulos and Crawford (3). In the experiments reported here, the class 2 (anthranilic acid-accumulating) mutants are placed between the class 1 and class 4 mutants, whereas the earlier studies place the class 2 types (mutants T1 and T11) between the CDR accumulators and the tryptophan synthetase mutants on the genetic map. This apparent anomaly may be due to the fact that there undoubtedly exist two phenotypically similar classes of mutants, those which are defective in the PR-transferase and PRA-isomerase functions. In this light, it seems likely that the anthranilic acid-accumulating mutants reported earlier [T1 and T11 (2), referred to as try 1 by Nester et al. (12)] correspond to the class 3 mutants of the present studies. This conclusion is supported by the finding that an extract of T11 does in fact lack PRA-isomerase activity (Whitt and Carlton, in preparation).

From these studies, it would appear that all of the gene functions concerned with tryptophan biosynthetic enzyme structure are localized in a tight cluster on the B. subtilis genome, since all six expected phenotypic classes of mutants have now been isolated and all of the mutational sites can be localized within the cluster. A definitive comparison of the genetic organization of this system with those described for the enteric bacteria is somewhat premature at this point because of the lack of conclusive evidence relative to the specific functions of the class 2 and class 4 mutants. If, however, these are assigned the PR-transferase and InGP synthetase functions, respectively, as seems most likely in view of the accumulation properties of the mutants, then the genetic organization of B. subtilis would be essentially analogous to that described for the enteric bacteria, in terms of the orders of the several cistrons. The final question, that of the possible operon nature of the system, likewise awaits more detailed biochemical and genetic investigations to determine the regulatory properties of the several enzymes in the system under repressing and derepressing conditions and the isolation of polarity-type and other regulatory mutants.

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Literature Cited