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Gene-Enzyme Relationships in Histidine Biosynthesis in *Bacillus subtilis*

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Mutants for 9 of the 10 steps in histidine biosynthesis have been isolated and identified by enzyme assay. Each locus has been mapped in relation to the *aro* cluster and to other histidine loci by deoxyribonucleic acid-mediated transformation. The genes which code for enzymes 3, 6, and 8 of the pathway are linked to the *aro* cluster. A major histidine linkage group is composed of the genes which specify enzymes 1, 2, 5, 7, and 10. The locus which codes for step 9 of the pathway is unlinked to any other identified *his* loci. The major histidine cluster is loosely linked to *cysB* and is unlinked to any of the loci concerned with aromatic amino acid biosynthesis.

Investigations concerned with the genetics of histidine biosynthesis in microorganisms have revealed numerous differences in the distribution of genes which specify the enzymes of the pathway. In *Salmonella*, the 10 genes are clustered in a single operon (3). Likewise, in *Staphylococcus aureus*, all of the genes map in a single cluster (13, 14). In *Streptomyces coelicolor*, five genes comprise a single cluster and three additional genes are unlinked (4). A genetic analysis of histidine-requiring mutants in *Pseudomonas aeruginosa* revealed five complementation groups (16). In *Saccharomyces lactis*, nine histidine-requiring mutants mapped at four loci (23). In the fungi *Saccharomyces cerevisiae*, *Neurospora*, and *Aspergillus*, histidine loci are located on different chromosomes with the exception of a single cluster of three genes (2, 4, 11). Complementation studies in yeast have led to the suggestion that these three genes represent an operon (11).

Initial mapping studies in *Bacillus subtilis* revealed that histidine loci comprise at least two linkage groups (10, 18). One histidine locus mapped within a linkage group concerned with the biosynthesis of aromatic amino acids. A second histidine locus was unlinked to the *aro* cluster. The present investigation is an extension of that work. We have isolated mutants for 9 of the 10 steps in the biosynthetic pathway (Fig. 1). Each locus has been mapped in relation to the *aro* cluster and to other hisitidine loci by deoxyribonucleic acid (DNA)-mediated transformation.

MATERIALS AND METHODS

Bacterial strains. All strains were obtained by treatment of WB746 with *N*-methyl-*N'*-nitroso-guanidine (1). WB746 is a spontaneous tryptophan-independent revertant of *B. subtilis* 168 (22).

Transformation procedures. Transformation was performed according to the procedure of Nasser and Nester (17). To minimize false linkage due to uptake of several DNA molecules, the values reported are those obtained at limiting DNA concentrations as shown by the proportionate drop in transformation frequency upon dilution of the DNA. DNA was prepared according to the method of Dubnau, Smith, and Marmur (9), and the DNA concentration was analyzed according to the method of Burton (5).

Mapping techniques. The determination of marker order for histidine loci linked to the *aro* cluster by transformation is based on the approach detailed by Carlton for fine structure mapping of the tryptophan region in *B. subtilis* (6, 7). Three-point crosses were carried out by using a closely linked gene *tyrA* as a reference marker (17). Doubly marked strains were constructed by transforming a *tyrA*$^-$ *trpC*$^-$ recipient strain with DNA from a strain carrying the desired histidine marker. Tryptophan-independent strains of the genotype *tyrA*$^-$ *his*$^-$ were selected. The assignment of marker order is based on a comparison of the frequency of prototrophic recombinants in a transformation cross between a donor DNA carrying a specific histidine locus and a recipient carrying both the reference *tyrA* marker and a second histidine locus. If the auxotrophic site in the donor is located between the two auxotrophic sites in the recipient, then a quadruple crossover is required to generate the prototroph. Consequently, the frequency of *tyrA*$^+$ transformants would be less than that of *tyrA*$^-$ transformants. If the auxotrophic site in the donor is located to the right of both auxotrophic sites in the recipient, then the prototroph results from a double crossover. In this case, the frequency of *tyrA*$^+$ would be greater than that of *tyrA*$^-$ recombinants (6, 7).

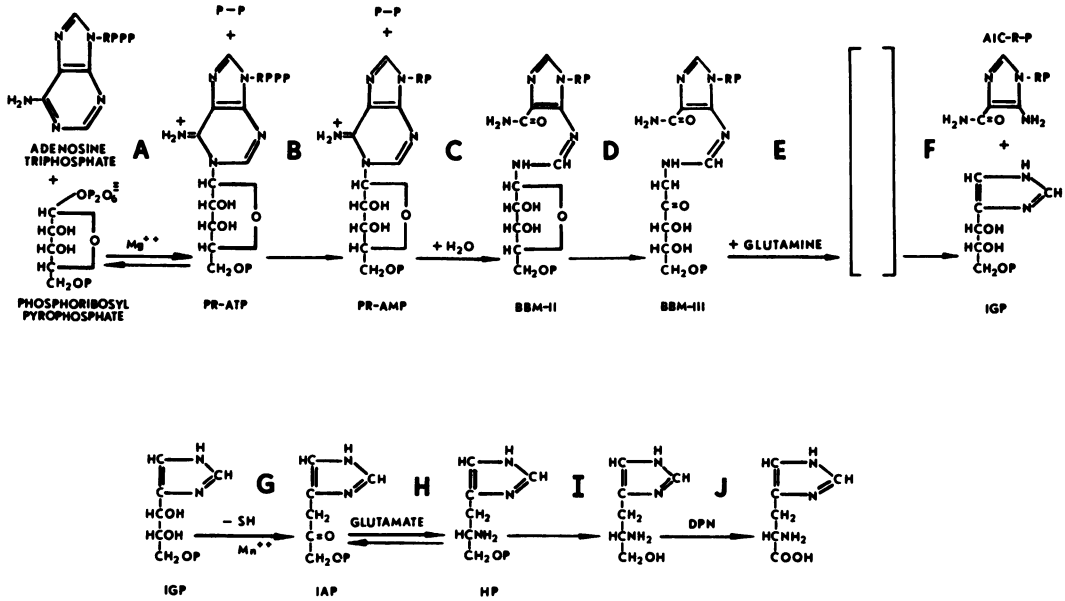

FIG. 1. *Histidine pathway as demonstrated in Salmonella typhimurium. The letters represent the genes which specify the enzymes in Bacillus subtilis. Abbreviations: PRPP, 5-phosphoribosyl-1-pyrophosphate; PR-AMP and PR-ATP, N-1-(5' phosphoribosyl) adenosine mono- and triphosphates; BBM, bound Bratton-Marshall compounds; AICR, 5 amino-1-(5' phosphoribosyl)-4-imidazolecarboxamide.*

Determination of recombination index. This technique is based on the prediction that the number of prototrophic transformants in a cross between two strains with identical nutritional requirements will be equal, regardless of whether the donor DNA was isolated from a prototroph or from a second histidine mutant if the mutation in the donor and the mutation in the recipient are unlinked. If, on the other hand, the mutant locus in the recipient and the mutant locus in the donor are linked, then a crossover must occur to generate the prototroph. Consequently, there will be fewer recombinants when DNA isolated from the mutant strain is used than when DNA from the prototroph is used. To correct for differences in transforming efficiencies, all results were normalized to the transformation of a marker unlinked to either the donor or recipient auxotrophic marker under study. We used *pheA* as the unlinked reference marker. Doubly marked *pheA*$^-$ *his*$^-$ strains were selected by transforming a *pheA*$^-$ *trpC*$^-$ recipient strain with DNA from strains carrying the desired his loci. The recombination index (R I) was calculated from the equation RI = (*loc m*/*ref m*)/*loc w*/*ref w*), where *loc* is the number of transformants to *his*$^+$ marker, *ref* is the number of transformants to *phe*$^+$, *m* is mutant DNA, and *w* is prototrophic DNA. A ratio of less than 1.0 indicates linkage.

Preparation of enzyme extracts. Growth of cultures, preparation of extracts, and assays for the histidine enzymes have been described previously (8).

RESULTS

The strains used in this investigation are described in Table 1. A strain was classified as a mutant for a given enzyme if the activity was negligible and a spontaneous single-step revertant regained the wild-type level of activity. Every mutant had wild-type levels of the remaining enzymes. In an analysis of approximately 200 histidine-requiring mutants, we obtained representative numbers of all enzymatic lesions, with two exceptions; only one strain lacked phosphatase activity and no isomerase mutants were found.

Linkage to the *aro* cluster was tested by determining the cotransfer of specific mutant loci with *tyrA* in a repulsion cross of the type *hisX*$^-$ *tyrA*$^+$ × *his*$^+$*tyrA*$^-$. The genes which code for hydrolase, cyclase, and aminotransferase activities are linked to the *aro* cluster (Table 2). Three-point crosses were carried out with *tyr-2* as a reference marker to determine the marker order for *hisC*, *his*–, and *his*$^+$ mutants. Results of crosses between representatives of three different linked genes are shown in Table 3. The donor mutant loci can be placed between the recipient markers when the *tyr*$^+$ frequency is low, or to the right of the recipient markers when the *tyr*$^+$ frequency is high. The genetic map shown in Fig. 2 represents the ordering of *his* loci linked to the *aro* cluster.

Non-linkage of his loci to independent aro genes. The genes which code for DAHP synthetase, dehydroquinase, DHS reductase, CHA synthase, and prephenic dehydratase are neither linked to the *aro* cluster nor to each other (17). We con-

TABLE 1. *Description of Bacillus subtilis strains used in the mapping experiments*

Strain	Enzymatic lesion
hisA579 *hisA580*	Phosphoribosyl transferase
hisB2363 *hisB2212*	Pyrophosphohydrolase
hisC838 *hisC2221*	Hydrolase
hisE2027 *hisE2234*	Amidotransferase
hisF2223 *hisF2211*	Cyclase
hisG947 *hisG2426*	Dehydratase
hisH2285 *hisH839*	Aminotransferase
hisI2376	Phosphatase
hisJ2235 *hisJ2242*	Dehydrogenase

TABLE 2. *Linkage of his loci to the aro cluster*[a]

Strain	Percentage of cotransfer
hisA579	0
hisA580	0
hisB2363	0
hisB2212	0
hisC838	57
hisC2221	69
hisE2027	0
hisE2234	0
hisF2223	61
hisF2211	62
hisG947	0
hisG2426	0
hisH2285	83
hisH839	91
hisI2376	0
hisJ2235	0
hisJ2242	0

[a] Linkage of his loci to the aro cluster was tested by determining cotransfer with tyrA in a repulsion cross of the type *hisX*$^-$ *tyrA*$^+$ × *hisX*$^+$ *tyrA*$^-$.

sidered the possibility that each independent *aro* gene is linked to a cluster of histidine genes. Donor DNA prepared from representative histidine mutants was used to transform reference mutants for each of the five independent *aro* loci. Transformants were selected on histidine-containing media. We determined the ratio of histidine-requiring to histidine-independent transformants. Those *his* loci unlinked to the *aro* cluster are not linked to any genes concerned with the biosynthesis of aromatic amino acids.

TABLE 3. *Ordering of histidine loci linked to the aro cluster in Bacillus subtilis*[a]

hisY$^-$ *tyrA*$^+$ donor	*tyrA*$^-$ *hisX*$^-$ recipient strains					
	C838	C2221	F2223	F2211	H2285	H839
C838....	0	10	25	12	5	1
C2221...	9	0	19	7	6	3
F2223....	90	95	0	10	12	6
F2211....	85	80	55	0	8	14
H2285...	81	85	60	65	0	51
H839....	98	92	50	55	15	0

[a] Transformants were plated on minimal and tyrosine-supplemented agar. Figures are the percentage of *tyrA*$^+$ recombinants in the *hisX*$^+$ *hisY*$^+$ recombinant classes. At least 200 transformants were analyzed in the determination of the ratio of *tyrA*$^+$ to *tyrA*$^-$ recombinants.

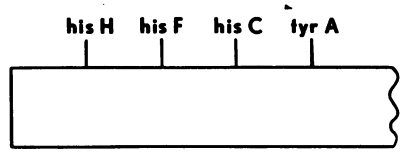

FIG. 2. *Histidine loci linked to the aro cluster in Bacillus subtilis. Ordering of these loci is based on data given in Table 3.*

Major histidine linkage group. Do the *his* loci which are unlinked to the *aro* cluster constitute a second histidine linkage group? We were unable to answer this question by Iscoring for recombination of a linked outside marker. Although *his* is loosely linked to *cysB*, as evidenced by 5% cotransfer at limiting DNA concentrations (Chapman and Nester, *unpublished data*), the two markers are probably too distant to be of use in mapping studies. We therefore determined recombination indices. DNA obtained from representatives of six different mutant classes was used to transform a *hisA580 pheA* recipient. The results shown in Table 4 indicate that four loci are linked to *hisA*. The phosphatase gene is unlinked to the major *his* cluster. The loci which are linked to *hisA* can be ordered as shown in Fig. 3.

DISCUSSION

The present investigation indicates that the pathway of histidine biosynthesis in *B. subtilis* is identical to that in *Salmonella*. However, the gene-enzyme relationships present a unique pattern among microorganisms. There are two linkage

groups, one composed of three loci linked to the *aro* cluster and a major histidine cluster consisting of five loci (Fig. 4). The phosphatase gene is not linked to either cluster. The physiological significance of this grouping is unclear. Although the relationship between gene order and biochemical sequence is not absolute in the *Salmonella* operon, in *B. subtilis* there is no discernible relationship between the order of the linked genes and the biochemical sequence.

The failure to obtain isomerase mutants despite the screening of a large number of the strains is difficult to explain. The mutant class has also remained unidentified in *Saccharomyces cerevisiae* and *Neurospora* (2, 11). Berlyn (4) has suggested that there are only two enzymes involved in the conversion of bound Bratton-Marshall compounds to imidazole glycerol phosphate in these organisms. Alternatively, the isomerase reaction may be catalyzed by a number of different enzymes if the reaction is nonspecific. A third possible explanation is that the isomerase activity is associated with another enzyme of the pathway and that a strain mutant for both components of the postulated aggregate is selected against under the conditions employed for the isolation of mutants.

The present investigation brings the number of genes in the *aro* cluster to 14. The histidine inhibition locus (*inh*), suppressor locus (*suh*), and 5-methyltryptophan resistance locus (*mtd*), previously described by Nester et al. (18), and the aminotyrosine resistance locus (*amt*), described by Polsinelli (19), most likely represent mutations in structural genes which result in modified feedback properties for a particular enzyme (Nester, *unpublished data*). The ordering of histidine loci which map within the *aro* cluster is based on three-point crosses and is unambiguous. The use of RI values to determine relative distances between linked loci which confer identical phenotype is based on the assumption that the frequency of histidine-independent transformants serves as a measure of the total number of recombination sites within a particular region of the bacterial genome (15). If the frequency of recombination between genes depends upon specific nucleotide modifications, as suggested by Ephrussi-Taylor (10*a*), then the use of RI values to order linked loci would not yield unequivocal results.

TABLE 4. *Recombination indices of reciprocal transformations between histidine-requiring mutants*[a]

hisX⁻ *phe*⁺ donor	*hisX*⁻ *pheA*⁻ recipient				
	his A580	his B2212	his E2027	his G947	his J2242
hisA580	0	0.50	0.10	0.05	0.20
hisA579	0.05	0.40	0.15	0.07	0.13
hisB2363	0.43	0	0.53	0.40	0.29
hisB2212	0.41	0.08	0.60	0.43	0.35
hisE2027	0.16	0.57	0	0.19	0.36
hisE2234	0.22	0.55	0	0.25	0.38
hisG947	0.09	0.44	0.18	0	0.13
hisG2426	0.16	0.37	0.20	0	0.05
hisJ2235	0.10	0.21	0.31	0.15	0.10
hisJ2242	0.20	0.30	0.37	0.20	0
hisI2376	0.86	1.1	1.0	1.0	1.1

[a] Transformants were plated on histidine-supplemented agar and phenylalanine-supplemented agar. The ratio of *his*⁺ to *phe*⁺ transformants was determined. The numbers in the table represent recombination indices which were determined as described in Materials and Methods.

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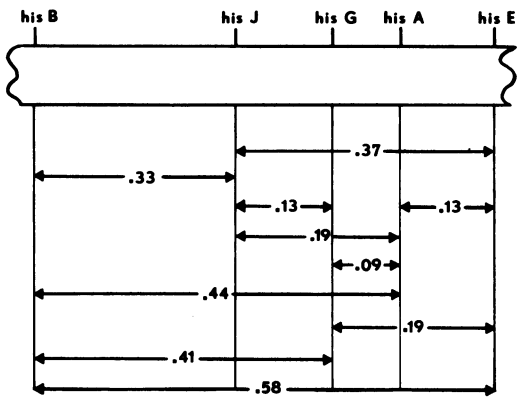

FIG. 3. *Ordering of loci in major histidine cluster in Bacillus subtilis. The distances are based on average recombination indices given in Table 4.*

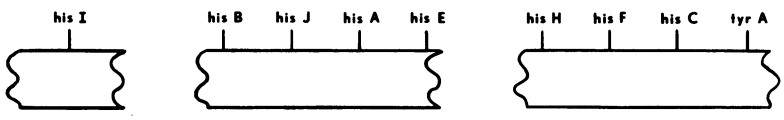

FIG. 4. *Two linkage groups concerned with histidine biosynthesis.*

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