

Genetics and Biochemistry of Pyrimidine Biosynthesis in *Bacillus subtilis*: Linkage Between Mutations Resulting in a Requirement for Uracil

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A number of independently derived uracil-requiring mutant strains of the parent strain 168 were mapped by inter-mutant transformation crosses. Only a few of these mutant strains were found to be transformable. Studies were performed in which these transformable uracil-requiring mutant strains were used as the recipient of deoxyribonucleic acid extracted from phenotypically similar mutant strains. The results yielded data resulting in a fine-structure map in which all mutations were found to be linked in a small region corresponding to that previously published as the uracil region of the *Bacillus subtilis* genome.

The majority of investigations which have contributed to our understanding of the relation between genetic constitution and biochemical activities of microorganisms have been performed with *Escherichia coli*. The results have been extrapolated to other organisms, prokaryotic and eukaryotic, and it is important that studies of similar systems, in at least a variety of unrelated microorganisms, be performed to assess the validity of such extrapolations.

It was our desire to compare a biochemical pathway in *Bacillus subtilis* with results obtained in similar studies in *E. coli* and other organisms. The pyrimidine biosynthetic pathway was chosen since it has been studied extensively in a variety of organisms and found to follow the same sequence of enzymic reactions (14). Also, linkage between four enzymes of this pathway was at one time reported in *E. coli* (1), but subsequent reports have indicated that only two of these four enzymes map close to each other (17, 18). All four enzymes were, however, subject to coordinate repression and derepression (1). In other organisms studied, no linkage has been found, while a variety of combinations of substrate induction, end-product repression, and no apparent regulation have been observed (14).

Studies on feedback inhibition of one of the individual enzymes concerned in pyrimidine biosynthesis in *B. subtilis*, aspartate transcar-

bamylase (ATCase) (2, 13), demonstrated that this enzyme is different structurally from the ATCase of *E. coli*. This suggested to us that *B. subtilis* might prove also to be different from *E. coli* in other modes of control of the enzymes in that pathway. A starting point for genetic mapping of pyrimidine-requiring mutants in *B. subtilis* was provided by the work of Dubnau et al. (5), in which two mutations resulting in a requirement for uracil were found to map very close together at the site indicated in Fig. 1 by the designation *ura*.

The "feasibility" of fine structure mapping by transformation in *B. subtilis* was demonstrated by Carlton (3), but correlation of genetic and biochemical studies is available only for a small number of biosynthetic pathways in this organism, e.g., tryptophan (19), histidine (4), and the aromatic amino acids (11), and for the histidine degradative pathway (18). It is only in the histidine degradative pathway that the coordinate control of the enzyme products of a group of closely linked genes has been demonstrated.

This investigation had as its purpose the mapping of a number of mutations in *B. subtilis* which result in a requirement for uracil, searching for the presence or absence of close linkage. The results are presented in this paper. The nature of the biochemical deficiencies in the mutants and the regulation of enzyme activity or synthesis will be reported separately.

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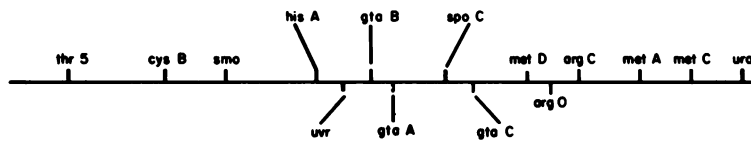


FIG. 1. Linkage of group III of the *Bacillus subtilis* genome. Adapted from F. E. Young et al. (21).

MATERIALS AND METHODS

Bacteria. Extensive attempts were made to isolate uracil-requiring mutants of *B. subtilis* 168MI⁻ after treatment of the strain with nitrosoguanidine or ultraviolet radiation. In all experiments performed, the number of potential mutants observed was extremely low, and in each case all isolates rapidly reverted to uracil independence. A number of *ura*⁻ derivatives of strain 168MI⁻ have been observed by other investigators. Of these, 14 were sufficiently stable for use in this investigation. The strains and investigators from whom they were obtained are listed in Table 1.

Media. Complex liquid medium was antibiotic medium no. 3 (A3, Difco Laboratories, Detroit, Mich.). Complex solid medium was tryptose blood agar base (Difco). Minimal medium consisted of Spizizen salts (16) supplemented with 0.5% glucose and 0.1% sodium glutamate. Nutritional requirements of the strains were added to give a final concentration of 50 μg/ml. Minimal and supplemented minimal media were solidified by adding purified agar (Difco) to give a final concentration of 1.5%. All media were prepared with glass-distilled water.

TABLE 1. *B. subtilis* strains used

Strain	Source	Requirements ^a
23W	J. Spizizen	None
168MI ⁻	J. Spizizen	Trp
12A	J. Spizizen	None (asporogenous)
168T ⁺	K. Bott	None
BK1	(23W—X168MI ⁻) ^b This study (168T ⁺ —X168MI ⁻)	None
168-15	W. Romig	Trp, Ura
168TUT	W. Romig	Trp, Ura, Thy
A26U	J. Marmur	Ura
SB5	E. Nester	Trp, His, Ura
SB8	E. Nester	Thr, Ura
SB270	E. Nester	Ura
SB305	E. Nester	Met, Ura
SB319	E. Nester	Met, Ura
WB577	E. Nester	Ura
17A-42	S. Greer	Ura
27-6	S. Greer	Ura
BR13	F. Young	Trp, Ura
BR72	F. Young	Trp, Ura
UTT	F. Young	Trp, Thr, Ura
AU	F. Young	Arg, Ura

^a Abbreviations used in designating the requirements are as follows: Arg, arginine; His, histidine; Ilv, isoleucine and valine; Leu, leucine; Met, methionine; Thr, threonine; Thy, thymine; Trp, tryptophan; Ura, uracil.

^b This notation indicates that DNA from strain 23W was used to transform the recipient strain 168MI⁻ (12).

Chemicals. All amino acids, pyrimidines and pyrimidine precursors used in these experiments were purchased from Calbiochem. Other chemicals used were of reagent grade quality.

Transformation. Cells were transformed by the method of Wilson and Bott (20). The regimen was standardized to obtain reproducibly high frequencies of transformation by selecting the most appropriate reagents. All media for transformation were prepared with distilled, charcoal-extracted, deionized water. When competent cells were to be frozen, cells were taken at the time of maximal competence, quick-frozen in the presence of 10% glycerol, and stored in liquid nitrogen. A concentration of deoxyribonucleic acid (DNA) (1 μg/ml), previously determined by us to be saturating, was used in all experiments.

DNA extraction. Transforming DNA was extracted from cells grown to the stationary phase in A3 medium. The cells were suspended in 0.15 M NaCl containing 0.1 M ethylenediaminetetraacetic acid and treated with lysozyme (1 mg/ml) in the presence of 20% sucrose, and the resulting protoplasts were lysed by treatment with 2% sodium lauryl sulfate and heated to 75 C for 10 min to destroy nuclease activity. The remainder of the procedure was essentially that of Marmur (10). The DNA concentration was determined by the modified diphenylamine colorimetric procedure of Giles and Myers (6), with calf thymus DNA as standard.

Construction of linkage map. Mapping was carried out by computing the recombination index (RI) (9) between mutants according to the following scheme. Cross 1 was, *ura*⁺*ref*⁺ DNA × *ura*₁⁻*ref*⁻ cells, and cross 2 was *ura*₂⁻*ref*⁺ DNA × *ura*₁⁻*ref*⁻ cells, where *ref* is the notation for the appropriate reference marker. RI = (number of *ref*⁺ transformants in cross 1/number of *ura*⁺ transformants in cross 1) × (number of *ura*⁺ transformants in cross 2/number of *ref*⁺ transformants in cross 2).

The designations *ura*₁⁻ and *ura*₂⁻ refer to any two different uracil-requiring mutants; as reference markers histidine, tryptophan, or methionine requirements were used, as appropriate, for the recipient strains. In this way, data can be corrected for the intrinsic variability of the transformation procedure.

RESULTS

Linkage experiments. Strain SB5 was transformed with DNA extracted from the wild-type strain BK1 and from each of the uracil-requiring strains. On different occasions, freshly prepared or frozen competent cells were used. In these experiments, the histidine requirement was used as the reference marker and, in addition, the tryptophan requirement was used

as a reference in those crosses in which it was applicable, i.e., in those crosses in which the donor strain did not have the tryptophan requirement. The strain BR13, a derivative of SB5, was also used as a recipient; however, recombination indexes were only computed for those crosses in which the donor strains did not require tryptophan since BR13 contains no other reference marker. Data obtained in one such experiment with freshly prepared competent cells of strain SB5 are presented in Table 2. The results recorded are indicative of those obtained in all experiments with the same recipient, but in this experiment the transformation frequency was high, allowing finer discrimination between closely linked mutations. The recombination indexes computed from these data are presented in Table 3 and represent composite figures from a number of experiments in which SB5 or BR13 were the recipients.

TABLE 2. Transformation of strain SB5

Donor	No. of transformants ($\times 10^6$)		
	<i>trp</i> ⁺	<i>his</i> ⁺	<i>ura</i> ⁺
BK1	13.7	28.0	27.0
SB5	0	0	0
SB8	11.0	13.0	3.96
BR13		22.0	0
17A-42	4.8	8.55	2.67
A26	11.2	27.3	1.57
BR72		21.6	5.46
SB270		16.3	0.97
SB305	15.4	24.9	0.14
SB319	18.0	40.8	8.7
WB577	8.6	19.3	5.9
168TUT		17.8	16.6
UTT		17.2	0
AU	22.7	20.1	0.116

TABLE 3. Recombination indexes in SB5 recipient crosses

Donor	RI
BK1	1.0
SB5	0
SB8	0.250
BR13	0
17A-42	0.305
A26	0.066
27-6	0.226
SB270	0.055
SB305	0.005
SB319	0.211
WB577	0.333
168TUT	0.965
UTT	0
AU	0.004

Strain SB319 was similarly transformed, and the data from these crosses are presented in Table 4. In this series of transformation experiments, the reference marker was methionine. The recombination indexes from these data are found in Table 5.

A comparison of the figures presented in Tables 3 and 5 demonstrates that mutations which map very closely to one of the recipients are all at approximately the same distance from the other. Strains SB270, SB305, A26, and AU, all of which map within less than 0.1 RI unit from SB5, are all at a distance of approximately 0.2 from SB319. On the other hand, SB8 and BR72, which are close to SB319, are at approximately the same distance from SB5.

Strain 168TUT in experiments performed with SB5 as recipient has consistently given results which suggest that it is only weakly linked to SB5, if at all. However, with strain SB319, the results suggest that linkage does

TABLE 4. Transformation of strain SB319

Donor	No. of transformants ($\times 10^6$)	
	<i>met</i> ⁺	<i>ura</i> ⁺
BK1	4.55	7.55
SB5	4.6	1.57
SB8	3.1	0.139
BR13	8.7	2.28
17A-42	1.1	0.206
A26	1.8	0.435
BR72	2.95	0.34
SB270	1.98	0.81
SB305	3.0	1.0
SB319	0	0
WB577	2.65	0.31
168TUT	4.08	2.96
UTT	4.3	0.7
AU	6.8	2.2

TABLE 5. Recombination indexes in SB319 recipient crosses

Donor	RI
BK1	1.0
SB5	0.206
SB8	0.027
BR13	0.158
17A-42	0.113
A26	0.145
BR72	0.07
SB270	0.245
SB305	0.205
SB319	0
WB577	0.07
168TUT	0.436
UTT	0.096
AU	0.195

exist between SB319 and 168TUT. Thus, it appears that 168TUT is weakly linked to SB5 and more closely linked to SB319.

The relative linkage distances between mutations which are derived from the computation of recombination indexes allow the construction of a fine-structure linkage map. Such a map of the uracil mutations investigated is presented in Fig. 2.

DISCUSSION

The genetic studies reported in this investigation have resulted in the construction of a fine-structure map for mutations having similar phenotypes, the requirement for uracil. The map consists of a cluster of six very closely linked mutations, probably alleles, at one end of the map, and a series of mutations at a distance from this cluster but on the same small DNA segment which are linked to one another in varying degrees. The biochemical nature of the lesion in these mutant strains will be presented in a separate report.

Use of recombination index to map *ura* strains. Attempts to obtain co-isogenic strains by introducing the uracil requirement into strain 168MI⁻ by transformation were unsuccessful. Potvin (personal communication) has recently shown that the selection of *ura*⁻ strains from the wild-type requires extreme selection pressure being accomplished only after repeated

applications of the penicillin selection technique. However, since all of the strains used in the present report were derivatives of strain 168, it was felt that areas of genetic nonhomology sufficiently large to obscure the results would not exist between them. In our studies, the use of the recombination index as a measure of the distance between closely linked genes has yielded results which are reproducible and, in general, give agreement in relative distances with different recipients. It should be pointed out, however, that it was found that the frequency of transformation to the reference marker had to be determined in *each* experiment.

In a previous report of linkage of loci governing the uracil requirement in *B. subtilis*, Dubnau et al. (5) mapped two of the uracil-requiring strains used in this study, SB5 and A26, by using the recombination index as used in our studies. When SB5 recipient cells were transformed with wild-type DNA and with A26 DNA with tryptophan as a reference marker, these workers reported an RI of 0.06. This figure is in agreement with our RI of 0.066 reported in Table 2.

It can be seen from the data presented in Table 2 that transformation of SB5 recipient cells with DNA from strain BR13 and strain UTT produced no transformants. That BR13 and SB5 possess the same mutation is known, since BR13 was derived as a *his*⁺ transformant

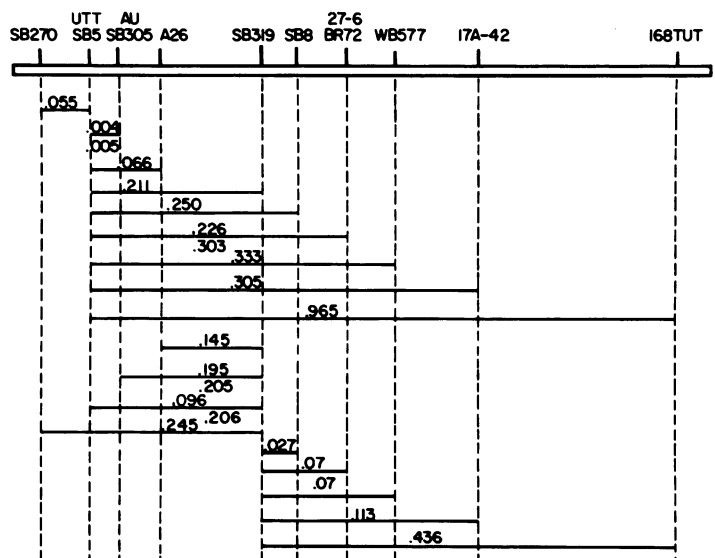


FIG. 2. Fine structure map of some pyrimidine-requiring mutants of *Bacillus subtilis*. The recombination index obtained from transformation studies is given as the distance between the markers. The upper nine distances are from data using SB5 as recipient, the lower nine using SB319. Where two strains are listed at one "locus," the upper recombination index on the line refers to the "upper" strain.

of SB5 (F. E. Young, personal communication). The data also suggest that the mutations in strains UTT and SB5 are extremely close together, if not identical, although data using SB319 recipient cells show a difference in RI for UTT and SB5 and place UTT immediately to the right of A26. Discrepancies of this sort point out the necessity of having supportive data in describing two mutations as identical using only one reference point in genetic mapping by transformation.

Orientation of ura loci with respect to other known loci. The present studies do not allow orientation of the uracil loci with respect to the rest of the genetic map. Dubnau et al. (5) reported linkage of the strain here designated A26 to a methionine marker at a distance which allows co-transduction by phage PBS1 at a frequency of 6%. With a co-transduction frequency of this low magnitude, it is unlikely that co-transduction with methionine would allow ordering of loci which are as closely linked as those reported here. Hoch and Anagnostopoulos (7) recently succeeded in isolating a recombination-deficient (*rec*⁻) mutant of *B. subtilis* (*recA*) which maps between uracil, the extreme right marker on linkage group III, and *argA*, the left marker on linkage group IV. It is possible to construct a single circular linkage map for *B. subtilis*, although the evidence is still inferential. This map is recorded by Young and Wilson (22) and lists the spore markers reported by Rogolsky (15) to be linked to a uracil marker.

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