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Cotransductional Mapping of the *trp-his* Region of *Bacillus megaterium*

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Eight *trp* mutations (four *trpE*, two *trpB*, one *trpC*, and one *trpD*) have been mapped in *Bacillus megaterium* QM B1551 and were found to be linked to two *hisH* mutations and unlinked to several other *his* mutations.

Bacillus megaterium is an industrially important organism which has been studied in several laboratories both because it is well suited for biochemical studies and because it undergoes 100% sporulation and synchronous germination during its cell cycle. Over the years, many interesting mutants have been isolated in this species, including some temperature-sensitive mutants blocked very early in germination (13) and a mutant resistant to oxidative phosphorylation uncouplers (7). Until recently, however, no genetic system was available to analyze these mutants. We have recently reported the isolation and characterization of a generalized transducing phage, MP13, for *B. megaterium* QM B1551 (14, 15) and preliminary crosses in the *leu-ilv* region (2). This region has now been more thoroughly mapped (J. C. Garbe, M. A. Franzen, and P. S. Vary, manuscript in preparation). We report here the mapping of two new regions, a cluster of *trp* genes cotransducible with *hisH* and an unlinked *his* region cotransducible with *azi*. All mutants were derived from prototrophic *B. megaterium* QM B1551 (ATCC 12872) and are listed in Table 1.

Conditions for cell growth, lysate production, and transductions were as previously described (15). Transductants were streaked for single colonies on the same selective medium, and two colonies from each streak were then tested by replica plating for unselected markers. Media employed included supplemented nutrient broth (10) and minimal glucose salt medium (6), omitting the Ca^{2+}, Mn^{2+}, and Fe^{2+} salts for transductions.

To determine growth requirements and gene defects, the *trp* mutants were plated on supplemented nutrient broth plates and replica plated to minimal medium supplemented with tryptophan biosynthetic intermediates anthranilate (10 μg/ml), indole (20 μg/ml), or L-tryptophan (20 μg/ml). The *trp* mutants were also streaked on minimal medium in close proximity to each other in every combination to test for cross-feeding ability. Growth was scored after 24 to 48 h. Accumulation of intermediates by the *trp* mutants was tested by growing the mutants in minimal medium plus 0.05% acid-hydrolyzed casein and 1 μg of tryptophan per ml (16) with shaking at 240 rpm and 30°C. After 24 h, the cultures were centrifuged at 12,000 × *g* for 10 min, and the supernatant was used for the following determinations. The presence of indole was assayed by a modification of the procedure of Yanofsky (17), substituting Kovac reagent for Ehrlich reagent. Indoleglycerol was detected using $FeCl_3$ reagent (18). The presence of anthranilate or 1-(*O*-carboxyphenylamino)-1-deoxyribulose was determined by the chromatographic procedure of Gibson et al. (3).

For cell-free extract preparation, cultures were grown to the mid-exponential phase in minimal medium plus 0.1% acid-hydrolyzed casein and 10 μg of tryptophan per ml, centrifuged, suspended in an equal volume of the same medium without tryptophan, and incubated with shaking for an additional 3 h. Cells were then harvested, extracts were prepared, and three enzyme levels as well as total protein were determined as described earlier for *Bacillus subtilis trp* mutants (5).

The biosynthetic defect could be tentatively assigned in seven of the eight mutants by these procedures. Mutants JV56, JV59, JV61, and JV62 grew on minimal medium plus anthranilate, indole, or tryptophan; could not cross-feed, but were cross-fed by the other mutants; and did not accumulate anthranilate, indole, indoleglycerol, or 1-(*O*-carboxyphenylamino)-1-deoxyribulose. These are, therefore, probably *trpE* mutants defective in anthranilate synthase, the first enzyme in the pathway. Mutant JV60 grew only on indole and tryptophan; cross-fed the *trpE*

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TABLE 1. *B. megaterium* mutants used in this study

Strain[a]	Genotype	Mutagenesis[b]
QM B1551	Prototrophic	
PV1	*hisH20*	NTG
PV3	*hisH21*	NTG
PV5	*cys-20*	NTG
PV44	*his-24*	EMS
PV139	*azi-1*	Spontaneous
PV140	*azi-2*	Spontaneous
PV141	*azi-3*	Spontaneous
JV56	*trpE1*	NTG
JV57	*trpB2*	NTG
JV58	*trpB3*	NTG
JV59	*trpE4*	NTG
JV60	*trpC5 str-3*	EMS
JV61	*trpE6 str-3*	EMS
JV62	*trpE7 str-3*	EMS
JV63	*trpED8*	NTG
JV65	*thr-2*	NTG
JV78	*leuC4*	EMS
JV80	*gly-2*	NTG
JV89	*his-4*	NTG
JV94	*his-9 str-3*	EMS
JV97	*his-12*	NTG
JV100	*ser-2 str-3*	EMS
JV109	*rib-2*	NTG

[a] All mutants with a PV prefix were isolated during this study. Those with a JV prefix were isolated by James C. Vary, University of Illinois Medical Center, Chicago, and were generously supplied by him. Only one of the strains, JV61, has previously been reported in the literature (12).

[b] NTG, *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine; EMS, ethyl methane sulfonate.

mutants but not JV57, JV58, or JV63; and accumulated 1-(*O*-carboxyphenylamino)-1-deoxyribulose. It was tentatively designated as *trpC*, defective in indoleglycerol phosphate synthase (11). Mutants JV57 and JV58 grew only on tryptophan, cross-fed all of the other mutants, and accumulated indole and indoleglycerol. This is characteristic of *trpB* mutants, which are defective in the B subunit of tryptophan synthase (8). One mutant, JV63, grew on indole and tryptophan, did not cross-feed the other mutants, and did not accumulate any detectable intermediate. Since it grew on indole and tryptophan but not on anthranilate, it could not be *trpE* or *trpB*. The rate of reversion in strain JV63 was found to be less than 6×10^{-8}, suggesting that the mutation in JV63 might be a deletion.

Enzymatic assays were performed on cell-free extracts of derepressed cultures of JV60 and JV63 to clarify the nature and extent of their genetic defect. The results (Table 2) confirm the assignment of JV60 to *trpC* and show that JV63 lacks phosphoribosyl transferase, the second enzyme of the pathway, but has excellent levels of the third and fourth enzymes of the pathway. Although we were unable to find conditions suitable for assaying the first pathway enzyme, anthranilate synthase, the failure of strain JV63 to accumulate anthranilate, coupled with the genetic evidence presented in Table 3, strongly suggests that it is a multisite mutation affecting both *trpE* and *trpD*. Pending further study, we will designate the lesion in this strain *trpED8*.

Histidine auxotrophs JV89, JV94, JV97, PV1, and PV3 were crossed with *trp* mutants JV57, JV58, and JV59 to test for cotransduction of *his* with *trp*. Only *his-20* of PV1 and *his-21* of PV3 showed cotransduction (50 to 65%) with the *trp* loci tested. All other crosses yielded no detectable cotransduction (<0.8%). In *B. subtilis* there are two known *his* loci, one at 305 on the revised map near *azi* and *cysB* and another, *hisH*, at 205 near *trp*, *ser*, *rib*, and *glyA* (4). The two *his* mutations near *trp* were tested for cotransduction with JV80 (*gly-2*), JV100 (*ser-2*), and JV109 (*rib-2*). Moreover, PV44 (*his-24*) was tested for cotransduction with PV5 (*cys-20*). None of these crosses gave positive results (<1%).

Histidine auxotrophs JV94 and JV97, which had exhibited no cotransduction with *trp*, were used as recipients in crosses with three mutants, PV139, PV140, and PV141, resistant to 1 mM azide, to determine whether these mutations were linked to *azi* as in *B. subtilis*. Cotransduction frequencies of 9 to 28% were observed between the two *his* auxotrophs tested and the azide mutants (data not shown). The tentative order was *his-9–his-12–azi*.

In an attempt to determine whether any of the histidine auxotrophs were either *hisH* or *hisD* (two defects which can readily be tested), all 12 *his* mutants were grown on histidinol (150 μg/ml) medium since *hisD* mutants fail to grow on histidinol (1). None were found to be mutant in *hisD*. All 12 *his* mutants were also tested for inhibition by *p*-fluorophenylalanine (*hisH*) to determine whether *hisH* or another *his* locus was linked to *trp* in *B. megaterium*. *hisH* mutants are deficient in aminotransferase, used also in the synthesis of phenylalanine, and thus are

TABLE 2. Enzyme assays of tryptophan auxotrophs

Strain	Enzyme activity[a]		
	Phosphoribosyl transferase	Phosphoribosyl-anthranilate isomerase	Indoleglycerol phosphate synthase
JV60	0.7	0.8	0
JV63	0	3.7	13.4

[a] Activity levels are the average of two independent determinations for phosphoribosyl transferase and one for the other two enzymes; they are expressed in millimoles per minute per milligram of extract protein.

TABLE 3. Transductional crosses between *trp* and *hisH*

Donor[a]	Recipient	Unselected/selected	% Cotransduction[b]
hisH21	*trpE1*	104/257	40
trpE4	*hisH20*	41/124	33
trpE4	*hisH21*	60/209	29
hisH21	*trpE4*	63/169	37
trpE6	*hisH20*	63/161	39
trpE6	*hisH21*	51/113	45
trpE7	*hisH20*	56/157	36
trpE7	*hisH21*	30/128	23
hisH21	*trpE7*	134/250	54
trpD8	*hisH20*	33/75	44
hisH20	*trpD8*	116/246	47
trpD8	*hisH21*	63/223	28
hisH21	*trpD8*	121/214	57
trpC5	*hisH20*	78/172	45
trpC5	*hisH21*	22/54	41
trpB2	*hisH20*	63/100	63
hisH20	*trpB2*	274/422	65
trpB2	*hisH21*	102/175	58
hisH21	*trpB2*	208/354	59
trpB3	*hisH20*	12/21	57
hisB21	*trpB3*	125/157	80

[a] Crosses were made as explained in the text, and when possible in both directions, although PV1 (*hisH20*) and JV58 (*trpB3*) were consistently poor donors.

[b] The percent recombination was calculated as 100% cotransduction.

more susceptible to the phenylalanine analog fluorophenylalanine (9). Sterile filter disks (12 mm) containing 0.1 ml of a 20-mg/ml solution of *p*-fluorophenylalanine were placed on soft agar (0.75%) overlays of each of the 12 *his* mutants. The zones of inhibition were scored for size and clearness after 24 h. Only the *his* auxotrophs PV1 (*his-20*) and PV3 (*his-21*), previously shown to be cotransduced with *trp*, were inhibited and therefore were probably *hisH* mutants.

Crosses between *hisH20* and *hisH21* and the *trp* mutants are shown in Table 3. Most reciprocal crosses were consistent, but two reciprocal crosses (*trpD8* and *trpE7*) with *hisH21* showed discrepancies. When *hisH21* was used as a recipient, less cotransduction was observed than when it was used as a donor. The average of the reciprocal crosses, however, was always consistent with the crosses of the *trp* mutant with *hisH20*. The largest discrepancy was observed in the *trpB3* crosses. It was very difficult to obtain a donor lysate of *trpB3*. The one successful experiment gave 57% cotransduction (Table 3) with *hisH20* as a recipient. However, when *hisH21* was used as a donor to *trpB3*, 80% cotransduction was found. The reciprocal cross was never successful, so we could not determine whether the same phenomenon seen with *trpD8* and *trpE7* was occurring. The consistent results observed in *trpB2* crosses with both *hisH* mu-

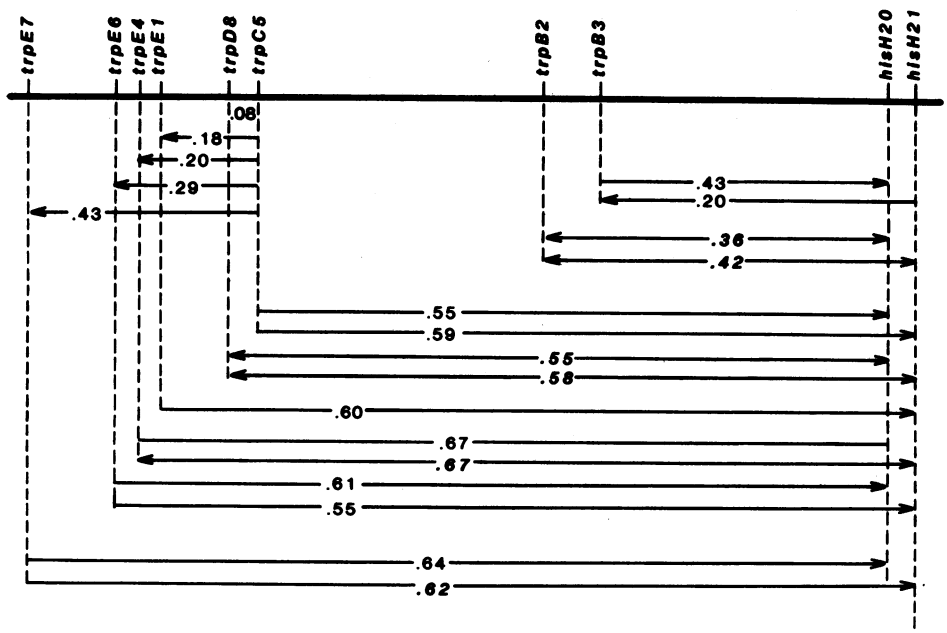

FIG. 1. Tentative map of the *trp-hisH* loci as determined by the data in Tables 3 and 4. In reciprocal crosses, the recombinational frequency shown is the average of the frequencies in each direction.

TABLE 4. Two-factor crosses to order tryptophan mutations

Donor	Recipient[a]					
	trpE1	*trpE4*	*trpE6*	*trpE7*	*trpD8*	*leuC4*
Wild type	65	118	80	97	58	54
trpC5	18 (0.82)	37 (0.80)	36 (0.71)	64 (0.57)	7 (0.43)	78
trpD8	1 (0.98)	1 (0.99)	10 (0.90)	3 (0.98)	0 (1.0)	66

[a] Crosses were performed to determine both the donor and the recipient abilities of the tryptophan mutants and the donor ability of the wild-type lysate. The top number in each cross is the average number of Trp^+ colonies present per plate, based on two to three trials. (Numbers are corrected for rate of recipient reversion.) The number in parentheses is the frequency of cotransduction, determined as follows: the expected number of prototrophs for each cross (E) was calculated as E = Md/Wd × Wm, where Md is the number of prototrophs observed using each *trp* mutant as a donor to the Leu^- recipient, Wd is the wild-type lysate crossed to the same recipient, and Wm is the number of prototrophs observed when the wild-type lysate was used as a donor to each *trp* mutant. The cotransduction frequency (CF) was then determined as CF = (E − O)/E, where O is the number of observed prototrophs in the tryptophan × tryptophan crosses.

tants probably are more reflective of the distance between the two *hisH* mutations and *trpB*. A tentative map of the *trp* mutants in relation to *hisH* based on the two-factor crosses is shown in Fig. 1. This is the first published map for *B. megaterium*.

The relative positions of the *trpE*, *trpD*, and *trpC* loci were determined through two-factor crosses (Table 4). These data are consistent with a multisite mutation of *trpD8* extending into *trpE*. The order of the mutations tested appears to be consistent with the gene order in *B. subtilis*, that is, *trpEDCFBA-hisH*, with another *his* locus near *azi*. Other markers which are proximal to *trp* in *B. subtilis* (e.g., *ser* and *rib*) have not been found to be linked in *B. megaterium*.

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ADDENDUM IN PROOF

Strain JV63 reverts on minimal + anthanilate medium. These revertants can then revert on minimal medium. Therefore, JV63 contains two point mutations, *trpD8* and *trpE29*, the latter which is not mapped.

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