NON-COORDINATE REGULATION IN 5-METHYL TRYPTOPHAN-RESISTANT MUTANTS OF BACILLUS SUBTILIS

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Investigations of the levels of tryptophan biosynthetic enzymes in 5-methyl tryptophan resistant mutants of Bacillus subtilis were undertaken in order to determine the effects of analog-resistance mutations on the regulation of tryptophan biosynthesis in this organism. The genes controlling each of the six functions necessary for the conversion of chorismic acid to tryptophan have been shown to map in a cluster in B. subtilis (Anagnostopoulos and Crawford, 1961, 1967: Carlton, 1967) as they do in Escherichia coli (Yanofsky and Lennox, 1959) and Salmonella typhimurium (Bauerle and Margolin, 1966; Blume and Balbinder, 1966). It was evident from this study, however, that they are not regulated in a coordinate manner.

In E. coli the tryptophan enzymes were shown to be repressed by tryptophan (Lester and Yanofsky, 1961), and in a coordinate manner (Ito and Crawford, 1965). These properties are characteristic of an operon-type of organization (Jacob and Monod, 1961). In addition to repression, the level of tryptophan produced is affected by the direct feedback-inhibitory action of tryptophan on anthranilate synthetase, the first enzyme specific for tryptophan biosynthesis (Gibson and Gibson, 1964; Moyed, 1960).

In B. subtilis, high levels of tryptophan have been shown to repress the synthesis of tryptophan synthetase. In addition, 5-methyl tryptophan blocks the formation of tryptophan (Nester, et al., 1963). As in the E. coli system (Cohen and Jacob, 1959), 5-methyl tryptophan resistance in B. subtilis was accompanied by elevated levels of tryptophan synthetase (Nester, et al., 1963).

Studies of the regulation of the tryptophan enzymes in a group of 58 B. subtilis tryptophan auxotrophs have proven difficult due to the multiple effects of many mutations on two or three of the enzymes of the pathway (Whitt and Carlton, 1968). For this reason the problem was approached by studying analog-resistant mutants.

MATERIALS AND METHODS

The chemicals used in this study were 5-methyl DL-tryptophan (Sigma Chemical Company), L-tryptophan (Calbiochem), and N-methyl-N'-nitro-N-nitrosoguanidine (Aldrich Chemical Company, Inc.). The characteristics of the strains which were used in this study are described in Table I.

When cell-free extracts were to be prepared, cells were grown for 15 to 18 hours on a rotary shaker at 37°C in 500 ml of each of three media: (a) a minimal salts medium (Spizizen, 1958) supplemented with 0.01% casein hydrolysate and 0.5% glucose, (b) the same medium further supplemented with 20 μg of L-tryptophan per ml, and (c) the same minimal medium supplemented with 25 μg of 5-methyl tryptophan per ml. Under conditions when whole cell assays were to be conducted, the cells were grown in 100 ml of each of the above media on a rotary shaker at 37°C for a period of seven hours (Whitt and Carlton, 1968).

To make extracts, cells were harvested by centrifugation at 13,000 x g, washed once in cold minimal medium, resuspended in 0.05M KPO_4 buffer at pH 7.5 containing 10^{-4}M EDTA, 0.5M KCl, 5 x 10^{-3}M 2-mercaptoethanol, and 30% glycerol, and subsequently disrupted in an Eaton pressure cell (Eaton, 1962). The glycerol was necessary for stabilizing phosphoribosyl-transferase and InGP synthetase activities in extracts; however, due to the interference of glycerol with the periodate reaction, it was necessary to measure the InGP synthetase activity in whole cells. The crude extract was centrifuged at 35,000 x g for 20 minutes to remove debris. The Lowry assay (Lowry, et al., 1957) was used for protein determination with bovine serum albumin as the standard.

The procedures for assaying anthranilate synthetase, PR-transferase, PRA-isomerase, InGP synthetase, and tryptophan synthetase B activities are described elsewhere (Whitt and Carlton, 1968). In addition to the regular reaction mixtures for each assay, extracts or whole cells were also assayed in a reaction mixture containing 20 μg of tryptophan per ml, and in a reaction mixture containing 100 μg of 5-methyl tryptophan per ml.

The extent of co-transfer between the tryptophan cluster and the mutations represented by the various methyl-tryptophan resistant mutants was estimated by two-point crosses using DNA prepared from the analog-resistant mutants and SB 194 as a recipient. The percentage of analog-resistant types in the prototrophic recombinant class was taken as the extent of linkage between the two sites.

RESULTS

When the levels of anthranilate synthetase were examined in cultures of 5-methyl tryptophan resistant strains grown on unsupplemented minimal medium, it was found that the different strains could be placed into three classes: (a) Group I strains were derepressed to a greater extent than wild-type cells; (b) Group II strains derepressed to approximately the same extent as wild-type cells; and (c) Group III mutants derepressed less than wild-type cells. The effects on the anthranilate synthetase activity of high levels of tryptophan and 5-methyl tryptophan in the growth medium of these strains are presented in Table II. Mutants in Groups II and III, those which were derepressed to levels equal to, or less than, the analog-sensitive prototroph, were all found to be repressible by tryptophan. In contrast, the mutants in

TABLE I

MUTANT CHARACTERISTICS

Strain	Properties	Source
SB 491	wild-type	Nester(Univ.of Washington)
SB 455	5 MT-resistant(1)	Nester(Univ.of Washington)
SB 194	Anthranilate synthetase$^-$	Nester(Univ.of Washington)
SP 1-SP 6	5 MT-resistant(1),spontaneous	SB 491
NG 107-NG 119	5 MT-resistant(1),NG-induced(2)	SB 491
NG 3	InGP synthetase$^-$,NG-induced(3)	W-1
NG 61	PR-transferase$^-$,NG-induced	SB 491
UV 15	InGP synthetase$^-$,UV-induced	W-1

(1)Resistant to 500 μg/ml of 5-methyl tryptophan

(2)Produced by a procedure similar to that described by Somerville and Yanofsky (1965) using NG 3 as a recipient in order to assure that the gene controlling 5-methyl tryptophan resistance would be linked to the tryptophan gene cluster.

(3)Sensitive to 500 μg/ml of 5-methyl tryptophan, but will grow on 25 μg/ml if 1 μg/ml of L-tryptophan is present. The properties of these strains have been described previously (Whitt and Carlton, 1968).

Group I, which were derepressed to a greater extent than the wild-type in minimal medium, were of two types -- either repressible or non-repressible by tryptophan. Within each of the three major groups some mutants were derepressed by 5-methyl tryptophan, others were repressed by the analog, and still others were unaffected by it. The presence of tryptophan or 5-methyl tryptophan in the growth medium appeared to have no significant effect on the growth rates of the 5-methyl tryptophan resistant strains. In contrast, the growth rates of the auxotrophic, 5-methyl tryptophan sensitive strains were stimulated by high levels of tryptophan, but were strongly inhibited by 5-methyl tryptophan unless a low level of tryptophan was included in the medium.

In all the mutants examined, both the 5-methyl tryptophan resistant prototrophs and the 5-methyl tryptophan sensitive auxotrophs, the addition of tryptophan to the reaction mixture caused a marked inhibition of the anthranilate synthetase activity similar to that of the wild-type enzyme. In a few of the mutants PR-transferase, PRA-isomerase, or tryptophan synthetase B appeared also to be somewhat inhibited, but not to the same extent as was the anthranilate synthetase activity. As in the case of added tryptophan, the addition of 5-methyl tryptophan to the reaction mixture caused inhibition of the anthranilate synthetase activity in all of the mutants examined, and partially inhibited

TABLE II

ANTHRANILATE SYNTHETASE LEVELS IN 5-METHYL TRYPTOPHAN-RESISTANT MUTANTS OF B. SUBTILIS**

Strain	Minimal medium	Minimal + 20μg/ml L-tryptophan	Minimal + 25μg/ml 5-methyl tryptophan
I. Derepressed more than wild-type on minimal medium			
A. Repressible by tryptophan			
NG 119	9.7	1.7	7.7
NG 61*	5.8	0	3.8
UV 15*	15.3	0	8.3
B. Non-repressible by tryptophan			
SP 1	16.7	11.9	15.0
SP 2	20.5	19.0	15.5
SP 5	14.5	13.5	18.0
SP 6	10.5	15.1	10.4
NG 108	7.7	8.4	8.4
NG 111	3.8	4.8	5.2
NG 113	10.9	7.1	7.3
II. Derepressed to same extent as wild-type on minimal medium			
SP 4	1.8	0.06	8.6
SB 491(w.t.)	1.6	0	2.9
NG 112	2.2	1.0	0.3
III. Derepressed less than wild-type on minimal medium			
NG 107	0.6	0	1.1
NG 114	0.1	0.1	0.1
NG 115	0.2	0.1	0.4
NG 116	0.2	0	0.1

*Methyl tryptophan sensitive tryptophan auxotrophs.
**Data presented as specific activities (mμ moles of anthranilate formed/minute/mg of protein).

the PR-transferase, PRA-isomerase, or tryptophan synthetase B activities in some strains.

The results obtained for enzyme assays of five of the six tryptophan enzymes from cells grown in maximally derepressing conditions are presented in Table III. As shown here, even in cases where anthranilate synthetase was highly derepressed, the other enzymes in the pathway were not necessarily derepressed, and in some cases most of the activities were derepressed less than wild-type.

TABLE III

RATIO OF MUTANT TO WILD-TYPE SPECIFIC ACTIVITIES

OF 5-METHYL TRYPTOPHAN-RESISTANT MUTANTS*

Strain	Anthranilate synthetase	PR-transferase	PRA-isomerase	InGP synthetase	Tryptophan synthetase B
I. Derepressed more than wild-type on minimal medium					
A. Repressible by tryptophan					
NG 119	6.1	2.4	5.9	1.1	5.0
B. Non-repressible by tryptophan					
SP 1	10.4	2.1	5.5	2.4	17.2
SP 2	12.8	1.9	8.2	3.3	11.4
SP 5	9.1	7.2	-	4.9	13.2
SP 6	6.6	1.1	7.0	5.5	11.8
NG 108	4.8	3.4	6.9	5.3	0.1
NG 111	2.4	2.5	6.6	1.6	0.4
NG 113	6.8	2.6	6.8	0.8	3.7
II. Derepressed to same extent as wild-type on minimal medium					
SP 4	1.1	0.3	1.8	0.6	0.5
NG 112	1.4	1.3	5.1	2.8	5.5
III. Derepressed less than wild-type on minimal medium					
NG 107	0.4	0.7	1.5	0.3	1.9
NG 114	0.1	0.1	1.0	1.0	3.0
NG 115	0.1	0.3	1.2	1.5	3.6
NG 116	0.1	0.1	0.8	1.9	2.6

*All cultures grown in Spizizen's minimal supplemented with 0.01% acid-hydrolyzed casein and 0.5% glucose.

Two-point transformation crosses with the various mutants gave co-transfer levels of the 5-methyl tryptophan resistance sites and the first gene in the cluster ranging from 1 percent to 60 percent. No correlation was observed between the degree of co-transfer and the extent of derepressibility.

DISCUSSION

In this study a number of 5-methyl tryptophan resistant strains were studied in an attempt to elucidate the means of regulation of the genes controlling the tryptophan biosynthetic enzymes in B. subtilis. The characteristics of these mutants, as summarized in Table II suggest that several different classes of 5-methyl tryptophan resistant mutants occur, thus leading to a variety of regulatory effects. The three major categories, reflecting

differences in the extent of derepressibility on minimal medium, probably represent the overall physiological effect of the mutational lesion on the ultimate level of active repressor in the cell. These classes probably correspond to the classes of 5-methyl tryptophan resistance mutations as recently described in E. coli (Hiraga et al., 1968). Since none of the 5-methyl tryptophan resistance mutant sites is transformed with a frequency of greater than 60 percent with the first gene in the cluster, it is unlikely that any of these represents an operator mutation.

The data presented in Table III on the enzyme levels produced by each of the mutants under optimal derepression conditions indicates that the regulation of the tryptophan enzymes in these analog-resistant mutants is non-coordinate, in that none of the strains examined shows derepression of all the tryptophan enzymes to the same extent. This conclusion assumes that the non-coordinate levels of enzymes observed are not due to a physiological pecularity of the cells in which some of the enzymes are preferentially degraded with respect to others. This possibility appears unlikely due to the wide variabilities observed for the relative levels of the various enzymes in the different strains examined. It should also be emphasized that the wild-type enzyme levels were measured in cells grown under derepression conditions, due to the fact that wild-type cells grown under conditions of repression have levels of enzymes that are not significantly above background. The conclusion that the expression of the tryptophan genes is non-coordinate is thus based on the comparative levels of derepression observed in 5-methyl tryptophan-resistant strains relative to wild-type, rather than the demonstration of non-proportional enzyme levels of wild-type grown under derepressing growth conditions.

This phenomenon of non-coordinate regulation might be brought about in several different ways. One is the existence of multiple operators, or sites of repressor action for each gene, each with a different affinity for a common repressor molecule. However, if this were the case, it would be expected that an alteration of the repressor-specifying gene, or any gene which affects the level of repressor, would result in proportional effects on the tryptophan enzymes, unless the mutation resulted in differential effects at the level of each operator-repressor affinity site. A second means of explaining this apparent non-coordinate regulation is to postulate the existence of multiple messenger molecules, and/or multiple sites of ribosome attachment, and/or multiple sites of initiation of polypeptide synthesis (e.g., one for each gene). If any of these is the case, one could get a non-proportional expression of the genes in the cluster. Although no direct evidence is available to substantiate or rule out these latter possibilities, the failure to obtain

polar mutants (those having a unidirectional effect on the genes in the pathway) in a series of 60 auxotrophic mutants induced with various mutagens (Whitt and Carlton, 1968) suggests that each gene may be expressed independently.

In conclusion, although the tryptophan genes map in a cluster in B. subtilis, as they do in the enteric bacteria, the results from this study suggest that the regulation of expression of these genes may be non-coordinate and suggest the possibility that the tryptophan genes of B. subtilis may not constitute an operon in the classical sense.

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