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Characterization of Mutants with Single and Multiple Defects in the Tryptophan Biosynthetic Pathway in *Bacillus subtilis*

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Sixty-five tryptophan auxotrophs which map in a cluster on the genome of *Bacillus subtilis* were characterized on the basis of (i) growth response, (ii) accumulation of intermediate compounds, and (iii) determination of enzymatic defects. They could be placed into six phenotypic classes. Certain of the mutants exhibited pleiotropic effects on more than one enzymatic activity in a manner different from those effects reported for the tryptophan pathway in other organisms. Invariably, mutations in the second gene, that coding for phosphoribosyl transferase activity, were found to lack the indoleglycerol phosphate synthase activity specified by the third gene in the cluster; however, this polarity did not extend to genes more distal in the cluster. Furthermore, mutations in the gene which codes for phosphoribosyl-anthranilate isomerase not only led to a loss of this enzyme but also to a loss of phosphoribosyl transferase and indoleglycerol phosphate synthase. In contrast, mutations in either of the loci coding for these latter functions had no apparent effect on isomerase activity. No polarity of the conventional type was found, e.g., none of the mutations in any gene led to polarized effects on the levels of the enzymes specified by the other genes of the cluster. These observations indicated a possible in vivo aggregation involving the transferase, isomerase, and synthase enzymes, with the isomerase acting as the "key" enzyme in the aggregate.

The conversion of chorismate to tryptophan in microorganisms has been shown to be mediated by six enzymatic activities (27). The chemical conversions in the pathway, as they have been elucidated in *Escherichia coli* (29), *Salmonella typhimurium* (5), and various fungi (15), as well as the enzymes catalyzing the reactions, are shown in Fig. 1.

The genes controlling the enzymes necessary to the biosynthesis of tryptophan map in a cluster on the genome and constitute an operon, as defined by Jacob and Monod (18), in both *E. coli* (16, 20, 29) and *S. typhimurium* (3, 5). Mutants with similar properties have also been shown to map in a cluster on the genome of *Bacillus subtilis* (1, 2, 6, 7). The purpose of this study was to elucidate the biochemical characteristics of a series of tryptophan mutants of *B. subtilis*. A preliminary report of this work has already been presented (D. D. Whitt and B. C. Carlton, Bacteriol. Proc., p. 137, 1968).

MATERIALS AND METHODS

Substrates and chemicals. Chorismate was prepared from the culture supernatant fluid of *Aerobacter aerogenes* strain 62-1 according to the method of Gibson (14) and was subsequently crystallized from ligroin. 1-(*o*-Carboxyphenylamino)-1-deoxyribulose 5-phosphate (CDRP) was synthesized chemically by the procedure of Smith and Yanofsky (22). 5-Phosphorylribose 1-pyrophosphate (PRPP) was obtained from the Sigma Chemical Co., St. Louis, Mo.

Strains. Sixty-five tryptophan auxotrophic strains were derived from the wild-type strain SB 491 (obtained from E. W. Nester, University of Washington, Seattle). The mutants were induced by ultraviolet light, nitrosoguanidine, ICR 170, ICR 191 (acridine half-mustards), nitrous acid, hydroxylamine, low *p*H, or ethylmethane-sulfonate (B. C. Carlton and D. D. Whitt, *in preparation*). Strain SB 455, a 5-methyl tryptophan-resistant strain, was obtained through the courtesy of E. W. Nester.

Growth conditions. To prepare cell-free extracts, cells were grown for 15 to 18 hr on a rotary shaker at 37 C in 500 ml of a minimal salts medium (24) supplemented with 1 μg of L-tryptophan per ml, 0.01% casein hydrolysate, and 0.5% glucose. This level of tryptophan was found to give maximal derepression of enzyme synthesis while still permitting growth of mutants with a doubling time of approximately 1 to 1.5 hr. The cells reached a density of about 5×10^8/ml. Although they were no longer in log

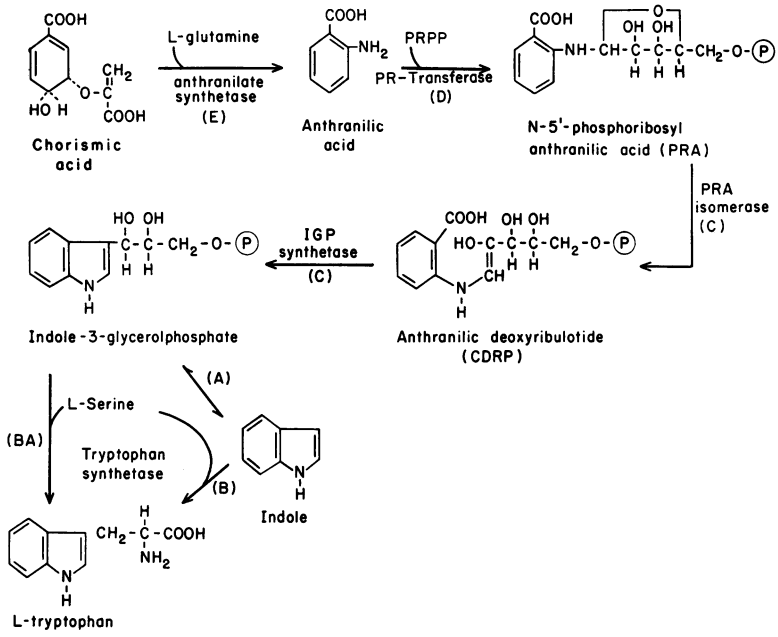

FIG. 1. *Pathway of tryptophan biosynthesis as determined for E. coli, S. typhimurium, and several fungi.*

phase, the cells grown for this length of time had approximately the same levels of activities for enzymes active in cell-free extracts as cells which were still actively dividing.

To conduct whole-cell assays, the cells were grown in 50 ml of the above medium on a rotary shaker at 37 C for 7 hr in order to obtain cells that were still in the log phase, since it was observed that the highest enzyme levels were obtained in actively dividing cultures. In this case, cells reached a density of approximately 2×10^8/ml.

Extraction of cells. Cells were harvested by centrifugation at 13,000 × *g*, washed once in cold minimal medium, resuspended in 0.05 M KPO_4 buffer (*p*H 7.5) containing 10^{-4} M ethylenediaminetetraacetate (EDTA), 0.5 M KCl, and 5×10^{-3} M mercaptoethanol, and subsequently disrupted in an Eaton pressure cell (11) at approximately −20 C. The crude extract was centrifuged at 35,000 × *g* for 20 min to remove debris. In some cases, the cells were disrupted by treatment with 1 mg of lysozyme per ml of cell suspension at 37 C until lysis occurred.

Protein assays. The Lowry assay (19) was used for protein determination with bovine serum albumin as a standard.

Growth response. To determine the growth requirements of the various mutants, these mutants were plated on nutrient agar plates (Microbiological Sciences, Inc., Yonkers, N.Y.) and then were replica plated to minimal agar plates supplemented with anthranilate (10 μg/ml), indole (20 μg/ml), or L-tryptophan (20 μg/ml).

Accumulation of intermediates. Mutant strains were grown for 24 hr in minimal medium supplemented with 0.05% casein hydrolysate, 1 μg of L-tryptophan per ml, and 0.5% glucose. The cultures were centrifuged, and the supernatant solutions were tested for the presence of various accumulation products. Indole was determined by the procedure of Yanofsky (25), and the presence of indoleglycerol was revealed by use of a $FeCl_3$ reagent (26). Anthranilate was identified by its high fluorescence in culture supernatant fluids and by its migration and fluorescence on paper chromatograms. The dephosphorylated form of CDRP was identified on chromatograms by staining with triphenyltetrazolium (22). Chromatographic separations were achieved by the method of Doy and Gibson (10).

Enzyme assays. Enzyme assays were conducted in one of two ways: whole cells and cell-free extracts. It was necessary to run whole-cell assays for some of the enzymatic activities because of their inactivity in cell-free extracts. Whole-cell assays were conducted for (i) phosphorylribose transferase (PR-transferase), which has never been found in cell-free extracts in these studies, (ii) indoleglycerol phosphate synthase (InGP synthase), which has also not been found in cell-free extracts, and (iii) for the conversion of indole to tryptophan (tryptophan synthase B). Cell-free extracts were used for the assay of anthranilate synthase and N-5'-phosphorylribose anthranilate isomerase (PRA-isomerase) and for the conversion of indole to tryptophan.

Anthranilate synthase. Anthranilate synthase was determined by measuring the appearance of anthranilate fluorometrically. The reaction mixture contained 2.8 μmoles of chorismate, 10 μmoles of $MgCl_2$, 10 μmoles of L-glutamine, 100 μmoles of KPO_4 buffer (*p*H 7.3), 5 μmoles of EDTA, plus extract and glass-distilled water to a final volume of 1 ml. Samples of

0.5 ml were taken at zero-time and after incubation at 37 C for 20 min. The reaction was stopped by addition of 0.05 ml of 1 N HCl. The anthranilate formed was extracted into 5 ml of redistilled ethyl acetate. Fluorescence was measured on a Turner model 111 self-balancing fluorometer. One unit of activity was defined as the amount of enzyme which formed 1 nmole of anthranilate in 1 min.

PR-transferase. PR-transferase was assayed in whole cells by measuring the disappearance of anthranilate fluorometrically. A whole-cell assay, involving a phosphorylated substrate (PRPP), was shown to be valid by use of appropriate controls, and by the observation that a strain which had activity when grown in derepressing tryptophan levels was inactive when grown in repressing levels of tryptophan. The reaction mixture contained 0.11 μmole of anthranilate, 0.36 μmole of PRPP, 5 μmoles of $MgCl_2$, 50 μmoles of KPO_4 buffer (*p*H 7.3), plus glass-distilled water to a final volume of 0.5 ml. This mixture was added to cells collected from 10 ml of growth medium. When mutants to be assayed were known from previous assays and accumulation studies to lack PRA-isomerase or InGP synthase activities, or both, the reaction mixture was also supplemented with an extract of strain T-80, an *E. coli* mutant lacking PR-transferase activity. (This supplied PRA-isomerase and InGP synthase functions to remove the PRA formed; otherwise, the PRA would have been broken down to anthranilic acid by the acidic extraction conditions employed.) After incubation at 37 C for 20 min, the reaction was stopped by the addition of 0.2 ml of 1 M sodium acetate buffer (*p*H 4.8). The anthranilate was extracted into 5 ml of ethyl acetate, and the fluorescence of the ethyl acetate layer was measured on a Turner fluorometer. One unit of activity was defined as the amount of enzyme capable of causing the disappearance of fluorescence equivalent to 1 nmole of anthranilate in 1 min.

PRA-isomerase. PRA-isomerase was detected in cell extracts by measuring the disappearance of anthranilate fluorometrically. The reaction mixture was the same as that used for measuring PR-transferase, with the exception that it was always supplemented with an extract of *E. coli* strain 9830, which lacks the PRA-isomerase function. The assay was performed in the same manner as the PR-transferase assay, with the exception that we used extracts rather than intact cells. In view of the variable levels of InGP synthase activities in the 9830 extracts used, quantitative assays of the isomerase activities in the *B. subtilis* extracts were not attempted.

InGP synthase. InGP synthase was assayed in whole cells according to a modification of the procedure of Smith and Yanofsky (23). This assay procedure was run with appropriate controls (i.e., zero-time samples and assays with no substrate) in order to rule out the possibility that a nonenzymatic release of accumulated indoleglycerol was responsible for the activity observed. The reaction mixture contained 0.22 μmole of CDRP, 50 μmoles of tris(hydroxymethyl)aminomethane (Tris)-chloride (*p*H 7.8), 2 μmoles of hydroxylamine (to prevent the further conversion of InGP to tryptophan), plus glass-distilled water to a final volume of 0.5 ml. This mixture was added to cells recovered from 10 ml of growth medium. After a 20-min incubation at 37 C, the suspension was treated with sodium metaperiodate for 20 min at room temperature to convert the InGP formed to indole-3-aldehyde. The indole-3-aldehyde was extracted into 5 ml of ethyl acetate, and the absorbance of the ethyl acetate was measured at 290 nm. A unit of activity was defined as the amount of enzyme which catalyzed the formation of 1 nmole of InGP in 1 min.

Tryptophan synthase B. Tryptophan synthase B component activity was assayed in extracts according to the method of Smith and Yanofsky (23). The reaction mixture for cell-free extracts contained 0.3 μmole of indole, 80 μmoles of DL-serine, 90 μmoles of Tris-chloride buffer (*p*H 7.8), 0.03 μmole of pyridoxal phosphate, 0.4 ml of saturated KCl solution [to enhance tryptophan synthetase B activity (21)], plus enzyme and glass-distilled water to a final volume of 1 ml. The reaction mixture for whole-cell assays, which were conducted according to the procedure of Eisenstein and Yanofsky (13), contained 0.3 μmole of indole, 80 μmoles of DL-serine, 0.03 μmole of pyridoxal phosphate, 50 μmoles of KPO_4 buffer (*p*H 7.8), and glass-distilled water to a final volume of 1 ml. This mixture was added to cells recovered from 10 ml of culture. A unit of activity was defined as the amount of enzyme which converts 0.1 μmole of indole to tryptophan in 20 min.

RESULTS

Three criteria were used to place the mutants into six groups: (i) growth response, (ii) accumulation products, and (iii) enzyme activities.

Growth response. The mutants were initially separated into three groups on the basis of their growth responses. The first group consisted of those mutants which grew on anthranilate as well as on indole and tryptophan. A second group of mutants responded to indole and tryptophan but not to anthranilate. This group contained all mutants blocked at any of the four steps between anthranilate and indole. The third group of mutants responded only to tryptophan.

Accumulation of intermediates. A second criterion for classifying the mutants was the identification of the intermediate compound accumulated by each mutant. On this basis, the mutants were placed into five groups. In the first group, there was no detectable accumulation in the culture supernatant fluids. These mutants responded to anthranilate as well as to indole and tryptophan. The second group of mutants accumulated a highly fluorescent compound which co-chromatographed with anthranilate. This group included mutants blocked presumably at either of the two steps between anthranilate and CDRP. The third group of mutants accumulated a less highly fluorescent compound,

which was assumed to be CDR on the basis of its migratory behavior on chromatograms and its reducing properties when treated with triphenyltetrazolium. The fourth group of mutants accumulated indoleglycerol (InG) in the culture supernatant fluid, as shown by a positive response to the $FeCl_3$ reagent. The last group of mutants, which accumulated indole (and, in some cases, InG as well), responded only to tryptophan.

Enzymatic activity. The third criterion for classifying the mutants was the presence or absence of specific enzymatic activities. The results of these experiments are presented in Table 1 and summarized in Tables 2 and 3.

All of the mutants in class 1 lacked anthranilate synthase activity in cell-free extracts. In addition, the activity of PR-transferase in whole-cell assays averaged only 66% of that of a wild-type strain grown under derepression conditions. On the other hand, InGP synthase activity in different mutants varied from 0.76 to 3.5 times that of the wild type, with an average of 2.7 times that of wild type, whereas tryptophan synthase B activity ranged from 1.5 to 2.6 times the wild-type activity in whole cells. All of these mutants had an active PRA-isomerase.

Mutants in class 2 completely lacked PR-transferase and InGP synthase activities in whole-cell assays. The level of anthranilate synthase varied from 0.3 to 6.1 times that of wild type in cell-free extracts, and the indole to tryptophan activity in whole-cell assays ranged from 2.0 to 3.4 times the wild-type level. These mutants had PRA-isomerase activity in cell-free extracts.

Class 3 is composed of mutants which had no demonstrable PRA-isomerase activity in cell-free extracts. These mutants were also defective in PR-transferase activity and InGP synthase activity (varying from 0 to 48% of the wild-type level) in whole-cell assays. Conversely, the anthranilate synthase levels were derepressed 1.6 to 5.6 times the wild-type level in extracts of different mutants, and the level of tryptophan synthase B activity in whole cells averaged 2.5 times the wild-type level.

All of the mutants constituting class 4 lacked InGP synthase activity in whole cells but had normal levels of PR-transferase activity (varying from 0.3 to 0.9 times that of the wild type). The tryptophan synthase B activity in whole cells averaged 2.5 times that of the wild type, and the level of anthranilate synthase activity in mutants of this class ranged from 1.8 to 8 times the wild-type level in cell-free extracts. These mutants exhibited PRA-isomerase activity.

Cell-free extracts of class 5a mutants, the InG accumulators, had PRA-isomerase activity and a level of anthranilate synthase which was activity. The levels of tryptophan synthase B

TABLE 1. *Summary of enzyme assays of whole cells and crude extracts*

Mutant	Specific activity[a]				
	Anthranilate synthase	PR-transferase	PRA-isomerase	InGP synthase	Tryptophan synthase B
Class 1					
UV 17	0	0.23	+	0.13	0.96
SB 194	0	0.85	+	0.45	0.93
ICR 1	0	0.31	+	0.28	0.71
ICR 2	0	0.44	+	0.54	0.99
ICR 3	0	0.43	+	0.52	1.00
ICR 4	0	0.50	+	0.58	1.14
ICR 5	0	0.25	+	0.42	0.85
ICR 6	0	0.63	+	0.53	1.01
ICR 14	0	0.43	+	0.51	1.01
ICR 16	0	0.65	+	0.56	1.24
Class 2					
NA 1	2.9	0	+	0	0.96
NA 3	6.9	0	+	0	1.06
NA 8	0[b]	0	+	0	1.17
NG 61	4.3	0	+	0	1.64
NG 62	5.0	0	+	0	1.27
NG 120	3.9	0	+	0	1.18
ICR 10	0.7	0	+	0	1.15
ICR 11	0.4	0	+	0	1.21
ICR 19	3.5	0	+	0	1.24
ICR 22	2.0	0	+	0	1.41
Class 3					
NA 2	0[b]	0.02	−	0.02	1.10
NA 4	3.5	0	−	0.04	1.15
NA 5	0[b]	0	−	0.08	1.68
NA 6	2.3	0	−	0	1.04
NA 7	2.3	0	−	0	1.18
NA 10	6.3	0	−	0.01	1.24
NA 11	3.9		−	0.03	1.23
HA 1	2.7	0	−	0.02	1.08
HA 2	0[b]	0	−	0.04	1.19
PH 1	0[b]	0	−	0.03	1.10
ICR 12	3.9	0	−	0.05	1.14
SB 11	1.8	0	−	0	0.70
Class 4					
NG 1	8.9	0.40	+	0	1.10
NG 2	6.6	0.20	+	0	1.15
NG 3	9.0	0.41	+	0	1.25
NG 5	7.5	0.38	+	0	1.33
NG 24	8.8	0.25	+	0	1.15
UV 15	7.4	0.60	+	0	1.29
UV 16	3.9	0.38	+	0	1.29
UV 18	5.2	0.62	+	0	1.28
168	2.2	0.50	+	0	1.03
EMS 1	9.2	0.31	+	0.02	1.19
EMS 2	2.0	0.55	+	0	1.33

0.03 to 5.8 times that of wild type. In whole-cell assays, the level of PR-transferase averaged 0.7 times that of the wild type, and the level of InGP synthase varied from 0.8 to 7.7 times the wild-type activity. The levels of tryptophan synthase B

TABLE 1—*Continued*

Mutant	Specific activity[a]				
	Anthranilate synthase	PR-transferase	PRA-isomerase	InGP synthase	Tryptophan synthase B
Class 5a					
NG 63	0.03	0.40	+	0.14	0
NA 9	3.3	0.58	+	1.04	1.00
T 50	0[b]	0.59	+	0.56	1.02
T 51	0[b]	0.62	+	0.71	0.13
ICR 7	6.6	0.40	+	1.28	0.02
ICR 17	0.8	0.54	+	1.17	0.05
ICR 21	2.5	0.16	+	0.21	0.04
Class 5b					
NG 4	3.2	0	+	0.47	0.04
NG 6	5.8	0.35	+	0.46	0.02
NG 7	3.9	0.31	+	0.79	0
NG 14	5.2	0.65	+	1.12	0
NG 23	5.2	0.35	+	0.60	0.01
NG 57	5.3	0.21	+	0.33	0.02
NG 58	5.9	0.68	+	0.73	0.01
NG 59	1.1	1.31	+	1.63	0.09
NG 60	3.4	0.58	+	0.79	0.03
ICR 8	5.2	0.37	+	0.84	0.02
ICR 9	0.3	0.85	+	0.59	0.04
ICR 13	3.2	0.55	+	1.15	0.04
ICR 15	4.3	0.71	+	1.06	0.07
ICR 18	7.3	0.64	+	1.17	0.08
ICR 20	5.3	0.57	+	0.59	0
SB 455	24.2	1.85	+	0.43	1.10
SB 491	1.13	0.71	+	0.17	0.48

[a] Specific activity for whole cell assays = no. of units/10^9 cells. Specific activity for crude extracts = no. of units/mg of protein.

[b] This activity was also absent, since these strains were isolated as double mutants carrying the SB 194 anthranilate synthase mutation.

varied from no activity to 2.2 times the wild-type activity.

Class 5b is composed of mutants which lacked the ability to convert indole to tryptophan in whole cells or in cell-free extracts. These mutants had PRA-isomerase activity and a level of anthranilate synthase 0.27 to 6.4 times the wild-type level, with an average of 3.8 times the wild-type level in cell-free extracts. In whole-cell assays, the level of PR-transferase was variable, ranging from 0 to 1.8 times that of the wild type, and the level of InGP synthase was derepressed 2 to 9.9 times the wild-type level.

SB 455, a 5-methyl tryptophan-resistant strain, had derepressed levels for all activities. Tryptophan synthase B was derepressed 2.3-fold; InGP synthase and PR-transferase were both derepressed 2.6 times the wild-type level. Anthranilate synthase was markedly derepressed to 21.4 times the wild-type level.

DISCUSSION

In this study, it was possible to classify a number of tryptophan auxotrophs into six different phenotypic classes, which correspond to the six classes of mutants described for other microorganisms (9, 27).

The relative orders of the mutant loci on the genome of *B. subtilis*, as reported previously (1, 2, 7), are: class 1, class 2, class 4, class 3, class 5b, class 5a. If this cluster of genes forms an operon in *B. subtilis*, one would expect to find polarity effects in certain mutants. For example, some mutants in the gene coding for anthranilate synthase might be expected to have polar effects on genes located distal to this region. However, none of the mutants examined in these studies displayed polarity of the conventional type. Those mutations in the first gene which led to lower activities of the products of certain other distal genes (InGP synthase and tryptophan synthase B) did not lower the function of the next adjacent gene, that controlling PR-transferase.

Mutations localized in the region coding for PR-transferase exhibited a pleiotropic effect, in that the next gene region distal to this region, the one which codes for InGP synthase, did not produce an active product when the PR-transferase gene was mutated. Pleiotropic effects were also observed in the PRA-isomerase (class 3) mutants. When mutations occurred in the region coding for this enzyme, the products of the two gene loci located proximal to it (InGP synthase and PR-transferase) were also rendered nonfunctional. Mutants in the InGP synthase region also enhanced the level of the product of the most proximal gene in the cluster, the anthranilate synthase locus.

One interpretation, suggested by the data presented in this paper, is that the PRA-isomerase, PR-transferase, and InGP synthase enzymes form an in vivo aggregate. The "key" enzyme in this aggregate appears to be PRA-isomerase, since mutations in the region coding for this enzyme invariably lead to loss of both PR-transferase and InGP synthase activities. However, the PRA-isomerase enzyme exhibits independence of the other two functions by remaining active in cell-free extracts, whereas the other two functions become inactivated.

In addition to their relationship to PRA-isomerase, there is also some interdependence of the PR-transferase and InGP synthase functions. As mentioned above, all of the PR-transferase mutants lack InGP synthase activity as well. From these observations, it appears that InGP synthase requires both an active PR-transferase and an active PRA-isomerase in order to be active itself. On the other hand, PR-transferase

TABLE 2. *Ratio of mutant specific activity to wild-type specific activity*[a]

Mutant class	Activities							
	Anthranilate synthase		PR-transferase		InGP synthase		Tryptophan synthase B	
	Range	Mean	Range	Mean	Range	Mean	Range	Mean
1	0	0	0.3–1.2	0.7	0.8–3.5	2.7	1.5–2.6	2.0
2	0.3–6.1	2.9	0	0	0	0	2.0–3.4	2.5
3	1.6–5.6	3.0	0	0	0–0.5	0.13	1.5–3.5	2.5
4	1.8–8.1	5.7	0.3–0.9	0.6	0	0	2.2–2.8	2.5
5a	0.03–5.8	2.3	0.2–0.9	0.7	0.8–7.7	4.4	0–2.2	0.7
5b	0.3–6.4	3.8	0–1.8	0.8	2.0–9.9	5.0	0	0

[a] Wild-type activity (SB 491) was set a value of 1.0. Comparative mean values for strain SB 455 were 21.4 (anthranilate synthase), 2.6 (PR-transferase and InGP synthase), and 2.3 (tryptophan synthase B)

TABLE 3. *Characteristics of tryptophan auxotrophs of B. subtilis*

Class	Mutant no.	Growth response	Accumulation product	Enzymatic defect
1	SB 194; UV 17[a]; ICR 1, -2, -3, -4, -5, -6, -14, -16	Anthranilate	None	Anthranilate synthase
2	NA 1, -3, -8[b]; NG 61, -62, -120; ICR 10, -11, -19, -22	Indole	Anthranilate	PR-transferase
3	NA 2[b], -4, -6, -7, -10, -11; HA 1, -2[b]; PH 1[b]; ICR 12; SB 11	Indole	Anthranilate	PRA-isomerase
4	NG 1, -2, -3, -5, -24; UV 15, -16, -18; 168; EMS 1, -2	Indole	CDR	InGP synthase
5a	NG 63; NA 9; T 50[b], -51[b]; ICR 7, -17, -21	Indole	Indoleglycerol	Tryptophan synthase A
5b	NG 4, -6, -7, -14, -23, -57, -58, -59, -60; ICR 8, -9, -13, -15, -18, -20	Tryptophan	Indole	Tryptophan synthase B

[a] Mutant designations refer to the mutagen used to induce the mutation. UV represents ultraviolet light, ICR refers to ICR 170 or ICR 191, NA represents nitrous acid, NG refers to nitrosoguanidine, HA is hydroxylamine, *p*H is low *p*H, EMS refers to ethylmethanesulfonate.

[b] NA 2, -5, -8; PH 1; HA 2; T 50 and -51 are double mutants carrying the SB 194 anthranilate synthase mutation as well as the mutation indicated in the above table.

apparently can function in vivo even though InGP synthase is inactive, providing the PRA-isomerase is normal.

It does not appear necessary for anthranilate synthase, PRA-isomerase, or tryptophan synthase B to be involved in the complex for activity in vitro. This conclusion is based on the small size and complete separability of each of these activities as elucidated by zone-centrifugation studies (*unpublished data*). On the other hand, the various pleiotropic effects observed may not be due to effects on aggregation, but may result from nonsense or frameshift mutations which have effects similar to polarity mutations of the conventional type (28). Although this does not seem likely, in view of the fact that polarity effects of the usual type were not observed, it is possible that this organism may contain natural nonsense suppressors which would prevent the expression of polarity mutants. However, no suppressors of this sort have been described in *B. subtilis*. Another factor which argues against this view is that the pleiotropic phenomena have been observed in all mutants of any particular class, regardless of the type of mutagen used to induce the mutation.

Finally, it is of interest to note that the single polypeptide InGP synthase-PRA-isomerase system as observed in *E. coli* (8) does not appear to exist in *B. subtilis*, since *B. subtilis* mutants which lack these two functions also lack PR-transferase. A difference in the organization of these enzymes, as compared to the enteric bacteria, is also supported by the observation that cell-free extracts of any mutant type or wild type which have normal levels of PRA-isomerase activity have no InGP synthase activity. This observation argues against both functions being carried out

by a single polypeptide. Furthermore, preliminary results obtained from zone-centrifugation studies indicate that PRA-isomerase is quite small (less than 30,000 molecular weight) and that the peak of activity has no detectable InGP synthase activity.

The dependence of anthranilate synthase upon PR-transferase as observed in *E. coli* (17), *S. typhimurium* (4), and *A. aerogenes* (12) appears likewise not to hold true for *B. subtilis*, since anthranilate synthase is active regardless of the presence or absence of PR-transferase activity. The small size of anthranilate synthase, as determined in zone-centrifugation experiments, also supports the idea that these two functions are not interdependent.

With regard to tryptophan synthase, the observation that some tryptophan synthase A mutants lack the ability to convert indole to tryptophan, whereas other A mutants have high levels of B activity indicates some degree of association between the two functions, although preliminary results on the separation of these two components suggest that association with the A component has relatively little influence on the B component activity.

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