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Tryptophan Biosynthesis in the Marine Luminous Bacterium *Vibrio harveyi*

C. D. BIEGER† AND I. P. CRAWFORD*

Department of Microbiology, University of Iowa, Iowa City, Iowa 52242

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Tryptophan biosynthetic enzyme levels in wild-type *Vibrio harveyi* and a number of tryptophan auxotrophs of this species were coordinately regulated over a 100-fold range of specific activities. The tryptophan analog indoleacrylic acid evoked substantial derepression of the enzymes in wild-type cells. Even higher enzyme levels were attained in auxotrophs starved for tryptophan, regardless of the location of the block in the pathway. A derepressed mutant selected by resistance to 5-fluorotryptophan was found to have elevated basal levels of *trp* gene expression; these basal levels were increased only two- to threefold by tryptophan limitation. The taxonomic implications of these and other biochemical results support previous suggestions that the marine luminous bacteria are more closely related to enteric bacteria than to other gram-negative taxa.

Understanding the natural (evolutionary) relationships among major bacterial taxa is one of the most challenging theoretical problems of modern microbiology, and methods for determining these relationships are not yet firmly established or agreed upon. The nature of this problem is well illustrated by the marine bacteria, organisms which share certain physiological characteristics, such as a tolerance of and requirement for sodium ions (4). Are the various genera possessing these properties closely related to each other, or do they instead merely represent halotolerant varieties of different well-studied terrestrial groups? The actual evolutionary relationships will probably be determined only through painstaking comparisons of findings, using a variety of methods and approaches.

Previously it was suggested that an examination of the genetic and biochemical features of the tryptophan pathway might yield insight into the natural relationships of bacterial groups (8). There are intragroup similarities with respect to gene arrangement, the presence of gene (and therefore polypeptide) fusions, and the pattern of regulation of the *trp* structural genes. Several biochemical properties of the *trp* enzymes also appear to be conserved. The differences among bacterial groups are substantial enough to allow logical conjectures about intergroup relatedness and possible lines of evolutionary descent. In particular, it has been suggested that the terrestrial enterobacteria are distinct from other bacteria in having all of their *trp* structural genes grouped in a characteristic operon and in exhibiting a fusion of *trpF* and *trpC*, genes for the third and fourth enzymes of the tryptophan pathway (Fig. 1).

Taxonomic studies of the marine luminous bacteria in which DNA-DNA hybridization, DNA-rRNA hybridization (4, 23), 5S RNA comparisons (28), immunological comparisons (2, 29), comparisons of selected enzyme regulatory patterns (3), and numerical taxonomy (including nutritional studies) (22) were used indicated that most of these bacteria can be placed either in the genus *Vibrio* or in the separate but related genus *Photobacterium* (5). Nevertheless, the taxonomic position of these organisms remains somewhat controversial. In *Bergey's Manual of Determinative Bacteriology*, 8th ed., all of the marine luminous bacteria are placed in the family *Vibrionaceae*, but the species formerly called *Beneckea harveyi* is assigned to the genus *Lucibacterium*. In this paper we use the binomial most recently proposed for this organism, *Vibrio harveyi* (5), recognizing that the same species, a ubiquitous free-living and marine enteric symbiont, has at other times been designated *Beneckea harveyi* or *Lucibacterium harveyi*.

Until 1974 no system of genetic exchange had been described for a luminous bacterial species. Then a specialized transducing phage for *V. harveyi* B392 capable of mobilizing the *trp* region was reported (16). The availability of this genetic vector suggested that biochemical and genetic comparisons might for the first time be feasible in a marine bacterial group.

We report here genetic, biochemical, and regulatory studies of the genes and enzymes of the

† Present address: Department of Biology, University of California at San Diego, La Jolla, CA 92093.

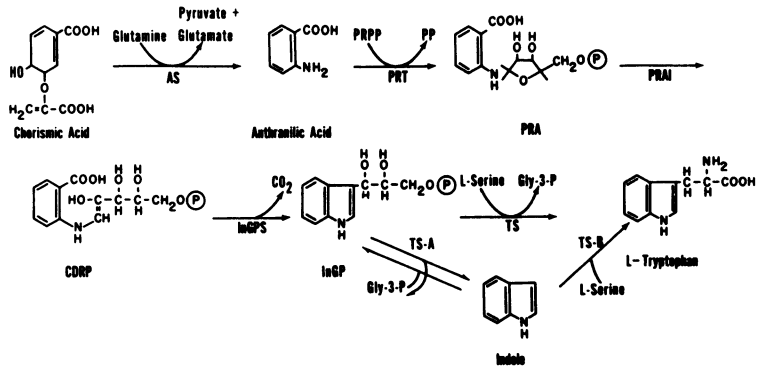

FIG. 1. Pathway of tryptophan biosynthesis. For AS, the physiological ASG reaction with glutamine as the donor is shown; this activity can be distinguished from the ammonia-dependent ASN activity. Abbreviations: PRPP, 5-phosphoribosyl pyrophosphate; PP, pyrophosphate; PRA, *N*-phosphoribosylanthranilate; CDRP, 1-(*o*-carboxyphenylamino)-1-deoxyribulose phosphate; InGP, indoleglycerol phosphate; Gly-3-P, D-glyceraldehyde-3-phosphate. See text for other abbreviations.

tryptophan synthetic pathway in *V. harveyi*. These studies include a description of the individual enzymes of the pathway and their coordinate regulation. Based on our results, it appears there are many similar features possessed by the terrestrial enteric bacteria and *V. harveyi*. Similar homologies exist in the genetic organization of the *trp* operon, as will be reported elsewhere (C. D. Bieger, I. P. Crawford, and K. N. Nealson, manuscript in preparation).

MATERIALS AND METHODS

Materials. Pyridoxal 5'-phosphate was purchased from Sigma Chemical Co. Phenylmethylsulfonyl fluoride (PMSF) was obtained from Pierce Chemical Co. Shikimic acid and tryptophan analogs were obtained from Sigma or Calbiochem.

Phage growth. *V. harveyi* strain B392 and phage hν-1 (16) were obtained from K. H. Nealson; preparation of phage stocks and details of transduction will be described elsewhere (Bieger et al., manuscript in preparation).

Media. The seawater complete (SWC) broth used was the complete medium described by Keynan et al. (16). Artificial seawater and basal medium (BM) were prepared as described by Reichelt and Baumann (22), except that the latter was supplemented with 0.3% glycerol, 1 μg of *p*-aminobenzoate per ml, and 0.05% acid-hydrolyzed casein (which lacks the amino acid tryptophan) unless otherwise noted. Growing overnight cultures in BM containing limiting (0.08%) glycerol (BML) was found to extend cell viability and reduce growth lag after resuspension in fresh medium; growth stopped at a density of 7×10^8 cells per ml in this medium. SWCT, BMT, and BMLT media were supplemented with 30 μg of tryptophan per ml. The growth of an auxotroph in BM was limited by tryptophan at a concentration of 7 μg/ml or below.

Preparation of extracts of derepressed cells. Cultures (100 to 200 ml) in BM containing 4 μg of tryptophan per ml inoculated with auxotrophs grown in BMLT were incubated overnight at 30°C with vigorous aeration in 1-liter flasks. At harvest samples were streaked onto mimimal medium plates to determine the proportion of Trp^+ revertants and tested for accumulation of intermediate metabolites. The cultures were centrifuged, washed twice with 50% artificial seawater, and suspended in 1.5 ml of cold 0.1 M potassium phosphate (pH 7.5) containing 20% glycerol. The cells were disrupted by sonic oscillation (two 30-s treatments), the lysates were cleared of debris by centrifugation for 45 min at 40,000 × *g*, and enzyme levels were assayed immediately. In certain experiments the cells were not washed. Instead, the cleared extracts were passed through columns (1.4 by 7 cm) of Sephadex G-25. Either procedure was satisfactory for removing intermediate metabolites. Repressed and partially derepressed extracts were prepared in a similar manner from cultures in BMT, BM, and BM containing indoleacrylate.

Derepression with indoleacrylic acid. To obtain values for the labile activity in the A half-reaction of tryptophan synthase (TS-A activity), a culture of strain NB2 growing in BM was made 20 μg/ml in indoleacrylate (100× stock was prepared in 50% ethanol), and a zero-time sample was removed immediately. This sample and each subsequent 80-ml sample was rapidly cooled to 4°C, and 0.05 volume of a solution containing 0.4% chloramphenicol, 1.3% sodium azide, and 2% $MgCl_2$ was added. The cells were centrifuged and washed with artificial seawater containing 0.04% chloramphenicol, 0.13% sodium azide, and 0.2% $MgCl_2$. The washed cell pellets were subjected to several freeze-thaw cycles by using a dry ice-acetone bath and then suspended in 1.5 ml of augmented sonication buffer (0.1 M potassium phosphate, pH 7.5, 20% glycerol, 1 mM PMSF, and 0.1 mM EDTA). After sonication and centrifugation to remove debris, enzyme assays were conducted as described below. Ames-Garry plots (enzyme activity versus anthranilate synthase [AS] activity) and Monod plots (enzyme activity versus culture turbidity) of derepression data were used initially, but the kinetic plots shown in Fig. 2 and 3 gave the best comparisons of derepression data.

TABLE 1. Strains of *V. harveyi* used in this work

Strain	Genotype[a]	Source or construction[b]
B392	Wild type (formerly MAV)	K. Nealson
NB2	*tna-2* (tryptophanaseless)	NG on B392
NB37	*tna*$^+$ *trpE37*	NG on B392
NB40	*tna-2 trpE40* (bradytroph)	NG on NB2
NB44	*tna-2 trpE44* (bradytroph)	NG on NB2
NB46	*tna-2 trpE46* (bradytroph)	NG on NB2
NB56	*tna*$^+$ *trpE56*	NG on NB2
NB162	*tna-2 trpE37*	NB37 (hν-1) × NB47
NB64	*tna-2 trpD76*	NB76 (hν-1) × NB47
NB76	*tna*$^+$ *trpD76*	NG on B392
NB154	*tna-2 trpD154*	NG on NB2
NB4	*tna*$^+$ *trpC4* (I$^-$) (formerly Trp-4)	NG on B392
NB9	*tna*$^+$ *trpC9* (I$^-$) (bradytroph)	NG on B392
NB34	*tna*$^+$ *trpC34* (I$^-$)	NG on B392
NB151	*tna-2 trpC151* (I$^-$)	NG on NB2
NB55	*tna*$^+$ *trpC55* (S$^-$)	NG on B392
NB62	*tna*$^+$ *trpC62* (S$^-$)	NG on B392
NB66	*tna*$^+$ *trpC66 ilv-1* (S$^-$)	NG on B392
NB51	*tna-2 trpC62* (S$^-$)	NB62 (hν-1) × NB47
NB36	*tna*$^+$ *trpC36* (I$^-$ S$^-$)	NG on B392
NB5	*tna*$^+$ *trpB5*	NG on B392
NB8	*tna*$^+$ *trpB8* (formerly Trp-8)	NG on B392
NB10	*tna*$^+$ *trpB10*	NG on B392
NB16	*tna*$^+$ *trpB16*	NG on B392
NB19	*tna*$^+$ *trpB19*	NG on B392
NB47	*tna-2 trpB47*	NG on NB2
NB38	*tna*$^+$ *trpA38*	NG on B392
NB63	*tna-2 trpA38*	NB38 (hν-1) × NB47
NB150	*tna-2 trpA150*	NG on NB2
NB155	*tna-2 trpA155*	NG on NB2
NB199	*tna-2 trp-1*	NG on NB2
NB203	*tna-2 trpE203 trp-1*	NG on NB199
NB206	*tna-2 trpD206 trp-1*	NG on NB199
NB200	*tna-2 trpC200 trp-1*	NG on NB199
NB207	*tna-2 trpB207 trp-1*	NG on NB199

[a] Strains with *trpC* mutations were characterized as lacking PRAI (I$^-$), InGPS (S$^-$), or both enzymes (I$^-$ S$^-$).
[b] For transductions, donor (hν-1) × recipient is shown. NG, *N*-methyl-*N*-nitro-*N*-nitrosoguanidine.

Enzyme assays. Both the amidotransferase (ASG) and aminotransferase (ASN) activities of AS were determined as described previously for *Acinetobacter calcoaceticus* (25). Anthranilate phosphoribosyltransferase (PRT) was assayed as described previously for *Escherichia coli* (15), except that the assay volume was 1 ml. Phosphoribosylanthranilate isomerase (PRAI) activity was determined as described previously (9). Indoleglycerol phosphate synthase (InGPS) was assayed by the method of Smith and Yanofsky (27) in 100 mM Tris buffer (pH 7.8). The activity of the B half-reaction of tryptophan synthase (TS-B; indole → tryptophan) was assayed in 1 ml of 90 mM potassium phosphate (pH 7.8) supplemented with 3% saturated NaCl, 20% glycerol, 30 mM L-serine, 10 μg of pyridoxal 5'-phosphate per ml, and 0.4 mM indole. Enzymic activity was stable for at least 2 h under these conditions. For estimations of the β_2-subunit, a threefold excess of the α-subunit (from either a *trpB* crude extract or a preparation of partially purified α-subunit) was added. Similarly, the α-subunit was measured with a threefold excess of the β_2-subunit by subtracting the activity found in the β_2-subunit control. TS-A activity (indoleglycerol phosphate → indole) was measured in 0.5 ml of a solution containing 100 mM potassium phosphate (pH 7.5), 20% glycerol, 3M salt-free NH_2OH (11), and 0.5 mM indoleglycerol phosphate (27). All incubations were at 37°C.

Mutagenesis and penicillin treatment. The strains used in this work and their derivations are shown in Table 1. Multiple 5-ml cultures of strain NB2 or B392 growing in BMT (doubling time, about 60 min) were incubated for 1 h with 10 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml, which resulted in approximately 10% survival, and then washed twice and grown overnight in BMLT. The next day the cultures were diluted 20-fold and allowed to grow for 4 to 5 h. A 2×10^{-3} dilution was made into BM, and 0.5 h later 5,000 u of penicillin per ml was added. Spheroplast formation was monitored microscopically, and when it was complete (1 to 2 h), osmotic shock lysis was accomplished by adding sterile water to 40% isotonicity. The surviving cells were collected by centrifugation and suspended in BMLT for overnight growth. Penicillin selection was repeated twice, and potential tryptophan auxotrophs were identified by replica-plating. After further strain purification and testing, one unique clone from each original culture tube was

selected, numbered, and stored on SWC slants at room temperature and in 30% glycerol at −20°C. Strains NB1 through NB117 were isolated by using 50 to 100 μg of nitrosoguanidine per ml, which resulted in 0.1 to 1.0% survival after mutagenesis; after this treatment the milder conditions described above were used to lessen the probability of nearby secondary mutations (20). Reconstruction experiments showed a marked decrease in auxotroph enrichment when the penicillin selection was conducted with more than 10^6 cells per ml (100-fold enrichment at a density of 5×10^5 cells per ml, 4-fold enrichment at a density of 5×10^7 cells per ml, and 2-fold enrichment at a density of 5×10^8 cells per ml), presumably due to cross-feeding of auxotrophs.

Isolation of tryptophanaseless strain NB2. The presence of the catabolic enzyme tryptophanase complicates growth and regulatory studies involving tryptophan by converting this compound to indole, pyruvate, and ammonia. This enzyme allows tryptophan to be used as the sole nitrogen source. *V. harveyi* cell density increased with increasing tryptophan concentration to 80% of the density in NH_4Cl-supplemented medium when the tryptophan concentration was 40 μM and then fell to 20% when the tryptophan concentration was 100 μM (presumably due to indole toxicity). No growth was observed when tryptophan was used as the sole carbon source (again probably due to indole toxicity). Although the use of phosphate to buffer marine media is usually contraindicated due to the copious precipitate formed, *V. harveyi* uses Tris as a nitrogen source, so a phosphaste-buffered minimal medium (10 mM potassium phosphate, pH 7.5, 22 g of sodium chloride per liter, 0.75 g of potassium chloride per liter, 0.1 g of $MgSO_4 \cdot 7H_20$ per liter, 5 mg of $FeSO_4 \cdot 7H_2O$ per liter, 3 ml of glycerol per liter, 0.05% acid-hydrolyzed casein, 1 mg of *p*-aminobenzoate per liter) was designed and found to be capable of supporting growth at a reduced rate (generation time approximately 200 min) for a limited number of passages. Selection for a tryptophanaseless strain was then accomplished as follows. Cells were mutagenized with 100 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml, grown overnight in SWC broth, and then diluted 20-fold into BM. When visible growth had occurred, the cells were harvested, washed, and suspended at a density of less than 10^8 cells per ml in phosphate-buffered minimal medium containing 40 μM tryptophan as the sole source of nitrogen. After 8 h of incubation, the culture was diluted to a density of about 10^6 cells per ml in the same medium containing penicillin, and incubation was continued for 6 to 8 h; this was followed by centrifugation and suspension in SWC medium. Penicillin selection was repeated three times and was followed by plating onto BM agar. Single colonies were picked and grown to stationary phase in SWC medium containing 40 μM tryptophan, and then samples were tested for the presence of indole. A mutant strain that could not produce indole from tryptophan was isolated and designated NB2 (*tna-2*). Attempts to isolate a tryptophanase deletion by using nitrous acid mutagenesis (26) followed by penicillin selection failed, probably due to the extreme sensitivity of this marine species to the brief period of low pH required during mutagenesis. However, we determined that strain NB2 reverted very infrequently and thus was suitable for subsequent work.

Isolation of regulatory mutant strain NB199. *V. harveyi* was tested for sensitivity to various tryptophan analogs; the order of inhibitory activity was determined to be 5-fluoroindole > DL-5-fluorotryptophan > DL-5-methyltryptophan > 5-methylanthranilic acid or 6-fluorotryptophan. Initial selections were made on minimal medium plates containing 10 to 200 μg of 5-fluorotryptophan per ml or 100 to 400 μg of 5-methyltryptophan per ml (the analogs were sterilized by filtration). Once strain NB199 had been isolated, we determined that plates containing both 15 μg of 5-fluorotryptophan per ml and 10 μg of shikimic acid per ml (7) were best for distinguishing between the *trp-1* mutation and its wild-type allele.

Cells were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and grown overnight in minimal medium, and 0.1-ml samples were plated onto analog-containing plates. Colonies appearing after 2, 3, and 4 days were picked and scored for constitutive production of the tryptophan enzymes by performing whole-cell assays for tryptophan synthase (TS) as follows. Tubes containing 9 ml of BMLT were inoculated and grown overnight to glycerol limitation; then the cells were centrifuged, washed in 50% artificial seawater, and centrifuged again, and the pellet was frozen for 5 min in a dry ice-ethanol bath and thawed for 5 min at 37°C. Samples (1 ml) of the usual TS reaction mixture were added, and the tubes were held in ice water while the cells were carefully suspended. After incubation at 37°C for 120 min, the indole remaining was determined colorimetrically as described above for the TS assays. Controls included wild-type cultures grown in the presence and absence of tryptophan and an auxotroph grown with limiting tryptophan; the difference between the repressed, basal, and derepressed enzyme levels of the controls was usually easily visible. All cultures showing any indication of derepression were purified and retested, and cell-free extracts of the one successful isolate (strain NB199) [*tna-2 trp-1*]) were shown to have elevated levels of all five enzymes of tryptophan biosynthesis whether the cultures were grown in the presence or absence of tryptophan.

RESULTS

Repressed, basal, and partially derepressed enzyme levels. The enzymes involved in the biosynthesis of tryptophan in *V. harveyi* were found to be regulated coordinately over a 100-fold range of enzyme specific activities. Table 2 shows the enzyme activities of prototrophic strain NB2 (tryptophanase negative) grown in minimal medium in the presence or absence of tryptophan. Although the extremely low levels of PRT and TS-A activities in a fully repressed extract made accurate measurements difficult, it appeared that all five enzyme activities were coordinately repressed to approximately one-fifth the basal level, which was defined as the activity in the wild type grown in minimal medium. This result differs from the results found with a number of enteric bacteria, where repression has been shown to be only semi-coordinate because of the presence of a low-level promoter in the distal one-third of the *trpD* gene (17).

TABLE 2. Enzyme levels in crude extracts of *V. harveyi* NB2

Enzyme	Activity (nmol/min per mg) under:[a]		Activity under repressed conditions/activity under basal conditions
	Repressed growth conditions[b]	Basal growth conditions[c]	
ASG	0.6	3.2	5
PRT	0.15	0.7	4
PRAI	0.75	3.6	5
TS-B[d]	1.3	6.5	5
TS-A[e]	0.035	0.13	4

[a] Average of three determinations.
[b] Cells were grown in BMT.
[c] Cells were grown in BM.
[d] Measured with excess α-subunit.
[e] Measured with excess β_2 subunit.

Partial derepression of the tryptophan enzymes can be achieved by using the tryptophan analog indoleacrylic acid (21), which seems to act by partially inhibiting the enzyme TS (16). Figure 2 shows the response of exponentially growing wild-type cells to the addition of indoleacrylate. All activities increased 5- to 10-fold during the first 1 h. Since PRAI and InGPS activities reside on the same polypeptide, we assumed that derepression of InGPS paralleled derepression of PRAI. Similar experiments in which early stationary phase cultures were used (data not shown) yielded similar results, although the activity change was neither as rapid nor as pronounced. The data plotted for TS-A activity were obtained in a separate experiment, after we learned that this activity is stabilized in buffers containing EDTA and PMSF. Since the TS-A values were determined with cells growing at 30°C, the time of sampling was normalized to the 25°C data by dividing by a factor of 2 (the difference in the growth rates at the two temperatures).

Enzyme activities in derepressed tryptophan auxotrophs. The results described above suggested that the five enzymes of tryptophan biosynthesis in *V. harveyi* are coordinately regulated over a 40- to 50-fold range in fully repressed, basal, or partially derepressed wild-type cells. Table 3 shows that a further increase in enzyme levels could be obtained when tryptophan auxotrophs were grown to a tryptophan limit. In these experiments the enzymes also appeared to be coordinately regulated. Table 3 also shows various classes of mutations within each *trp* gene.

V. harveyi TS is probably present as a multimeric complex (possibly $\alpha_2\beta_2$) derived from two genes, *trpA* and *trpB*. This arrangement has been observed in all bacteria examined to date (8). Although the *trpA* gene product is primarily responsible for the TS-A activity and, similarly, the *trpB* gene product is primarily concerned with the TS-B reaction, both subunits are required for full activity in either half reaction. Thus, a nonsense mutation in *trpA* that eliminates the α-subunit and abolishes TS-A activity may also be expected to reduce the activity of the TS-B reaction greatly. Such mutants have been called α CRM-minus, implying the absence of cross-reacting material in an immunological test designed to detect the presence of mutant α-subunits (10). β CRM-minus mutations act in an analogous manner to reduce TS-A activity, as well as abolish TS-B activity.

V. harveyi trpA and *trpB* mutations can be divided into two classes, those which simultaneously eliminate the activity of the other TS half-reaction (CRM-minus mutations) and those with relatively little effect on the complementary half-reaction (CRM-plus). The mutations of strains NB150, NB155, NB10, and NB19 are examples of the former, whereas the mutations of strains NB38, NB63, NB5, NB8, and NB47 are examples of the latter. Cell-free extracts of CRM-minus strains are valuable in assays of the levels of the absent subunit produced by other strains, for they permit the addition of saturating levels of one normal subunit without the necessity of purifying that subunit.

The *trpC* mutants, which will be described more fully elsewhere (Bieger et al., manuscript in preparation), can be divided into the following

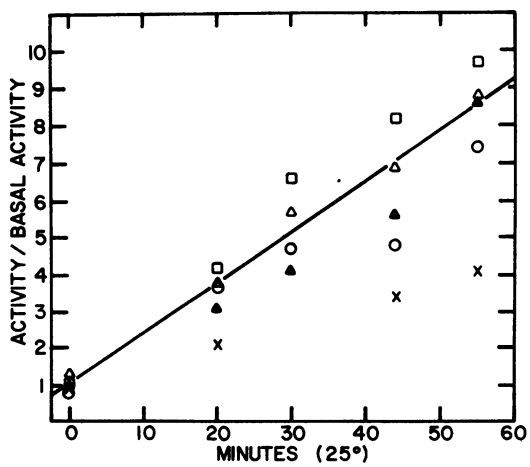

FIG. 2. Coordinate derepression of five tryptophan synthetic enzymes evoked by indoleacrylate. Wild-type cells exponentially growing in BM were exposed to indoleacrylate and analyzed as described in the text. The line shown was calculated by linear regression, using the data for the first four enzyme activities. The TS-A data were corrected for a different growth temperature (see text). Symbols: ○, ASG; △, PRT; □, PRAI; ▲, TS-B; ×, TS-A.

TABLE 3. Enzyme levels in extracts of cells grown to a tryptophan limit

Strain	Activity (nmol/min per mg)					
	ASN	ASG	PRT	PRAI	TS-B[a]	TS-A[b]
NB40 (*trp-40*)	3	6	1	19	15	0.2
NB44 (*trp-44*)	6	10	2	15	11	0.2
NB46 (*trp-46*)	2	4	1	23	14	0.2
NB37 (*trpE37*)	<0.2	<0.2	<1	104	60	
NB56 (*trpE56*)	<0.2	<0.2	26	118	105	2.3
NB162 (*trpE37*)	<0.2	<0.2	2	75	65	1.5
NB64 (*trpD76*)		62	<1	74	18	0.7
NB76 (*trpD76*)		126	<1	93	125	
NB154 (*trpD154*)	31	52	<1	46	35	0.6
NB4 (*trpC4*)	44	123	29	<1	114	2.7
NB9 (*trpC9*)	42	66	16	<1	35	0.9
NB34 (*trpC34*)		96	25	<1		
NB36 (*trpC36*)	27	46	20	<1	63	1.4
NB51 (*trpC62*)		75	26	98	70	
NB55 (*trpC55*)	34	53	10	21	24	0.9
NB151 (*trpC151*)	29	56	33	<1	35	0.6
NB5 (*trpB5*)		51	18	51	<1	1.0[c]
NB8 (*trpB8*)	30	104	20	133	<1	0.5 (1.5)[c]
NB10 (*trpB10*)		42	12	63	<1	<0.1[c]
NB19 (*trpB19*)		38	15	54	<1	<0.1[c]
NB47 (*trpB47*)		72	10	81	<1	1.0[c]
NB38 (*trpA38*)		48	6	50	35	<0.1[c]
NB63 (*trpA38*)		75	42	47	42	<0.1[c]
NB150 (*trpA150*)		101	18	230	33[d]	<0.1[c]
NB155 (*trpA155*)		55	20	142	19[d]	<0.1[c]

[a] Measured with excess α-subunit.

[b] Measured with excess β_2-subunit, except as noted below.

[c] Assayed without the addition of excess β_2-subunit. To obtain the value in parentheses, partially purified β_2-subunit was used instead of a crude extract of a *trpA* mutant as β_2 source.

[d] Very low levels of activity were found in the absence of added α-subunit.

three classes: those which have InGPS activity but are unable to catalyze the PRAI reaction (strains NB4, NB9, NB34, and NB151), those without InGPS activity but with PRAI activity (strains NB51, NB55, and NB66 [data not shown]), and those lacking both enzyme activities (strain NB36 and possibly strain NB200). As in *E. coli* and other enteric organisms, in *V. harveyi* both the PRAI and InGPS activities reside on the same polypeptide chain; therefore, these results are easily explained as being due to missense or nonsense mutations in a bifunctional polypeptide.

Strains containing lesions in *trpE* fall into two classes. The first (strains NB56 and NB162) shows little or no AS activity but reasonable levels of the other gene products. The second class consists of a single strain (strain NB37), which has simultaneously lost the PRT function (perhaps it is a *trpE trpD* double mutant). A group of three mutants (strains NB40, NB44, and NB46) has a peculiar phenotype; all are leaky when they are plated onto minimal agar and show uniformly low levels of activity (10%) of all five tryptophan biosynthetic enzymes. The pattern observed with these three mutants may be the result of some kind of regulatory mutation that affects all five structural genes in a similar manner.

Coordinate elevation of enzyme activities in strain NB199 containing a regulatory mutation. Strain NB199 was the sole derepressed mutant found after we tested 500 clones selected for resistance to the analog DL-5-fluorotryptophan. Selection for resistance to this analog has in the past proved to be valuable in the isolation of *trpR* (repressorless) and *trpO*^{c} (operator-constitutive) mutations (1, 13). Table 4 shows the insensitivity of the *trp* genes in this strain to repression by exogenous tryptophan. The activities were maintained at a constant level well above the basal level of activity of the wild type but somewhat below the activity of a fully derepressed auxotroph. Figure 3 shows that strain NB199 is capable of further derepression; two- to threefold increases were observed during the first 30 min after the addition of indoleacrylate. We constructed double mutants having the regulatory mutation (*trp-1*) of strain NB199 and a *trp* structural gene mutation; these mutants responded to tryptophan deprivation by a two- to threefold additional derepression of the

TABLE 4. Enzyme levels in strains containing *trp-1*

Strain	Tryptophan concn (μg/ml)[a]	Activity (nmol/min per mg)					
		ASG	PRT	PRAI	InGPS	TS-B	TS-A
NB199 (*trp-1*)	30	26	8.3	69	8	37	0.55
	0	26	7.7	75	8	43	0.65
NB200 (*trpC200 trp-1*)	30	19	8	<1		23	
	4	96	27	<1		55	
NB51 control (*trpC62*)	4	75	26	98		70	
NB206 (*trpD206 trp-1*)[b]	30	16		43	6		
	4	74		88	9		
NB207 (*trpB207 trp-1*)[b]	30	15		38	6		
	4	67		75	10		

[a] Level of tryptophan present in growth medium.
[b] Extracts were prepared and assayed in the absence of glycerol.

unaffected enzymes of the pathway (Table 4). The enzyme level observed did not exceed that found in comparable single auxotrophs, however. The mutation of strain NB199 was tentatively classified as a *trpO*^{c} (operator-constitutive) lesion on the basis of its contransducibility with the *trp* structural genes and its ability to undergo further derepression, but these two characteristics are not by themselves diagnostic; since definitive *cis-trans* dominance tests cannot be performed at present, the mutation has been designated *trp-1* instead of *trpO1*.

Although the results described above did not distinguish whether the strain NB199 mutation alters a repression mechanism or an attenuation mechanism, they did clearly indicate the presence in *V. harveyi* of a mutable regulatory region which simultaneously and coordinately affects the expression of all five enzymes of the *trp* gene cluster.

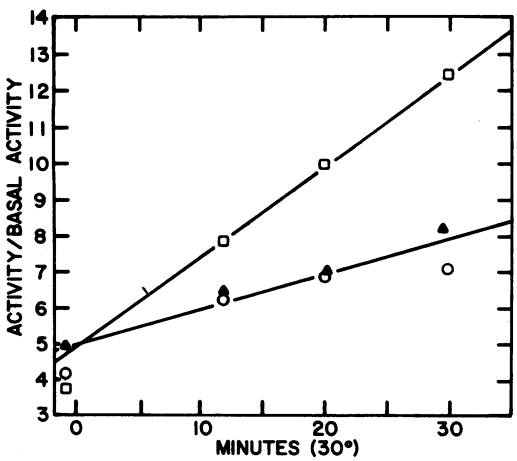

FIG. 3. Indoleacrylate derepression of the elevated basal enzyme levels in strain NB199. Symbols: ○, ASG; □, PRAI; ▲, TS-B.

Enzyme aggregation and molecular weights. Characterization of the tryptophan biosynthetic enzymes has been useful in the classification and comparison of different bacteria. For example, in the so-called "type 1" enteric organisms (including *E. coli*, *Salmonella typhimurium*, *Enterobacter aerogenes*, and *Erwinia dissolvens*), AS and PRT activities are associated in a multimeric complex having a relatively high molecular weight (18). Not only are both components necessary for full AS function, but also (in *E. coli* and *S. typhimurium* at least) the association of AS and PRT in a complex causes the PRT activity to become sensitive to feedback inhibition by tryptophan.

V. harveyi resembles the "type 2" enteric bacteria because only AS is affected by feedback inhibition (Fig. 4). Gel filtration confirmed that the AS and PRT activities are on separate protein molecules (Fig. 5A), and a comparison with protein standards provided molecular weight estimates of approximately 160,000 for AS and 65,000 for PRT. In this experiment the PRAI activity (data not shown) eluted at a position corresponding to a molecular weight of 45,000, whereas the TS-B activity was heterodisperse. One possible interpretation of the TS-B profile identifies the activity peaks (in order of decreasing molecular weight) as due to $\alpha_2\beta_2$, β_2, and β forms of TS. TS-A activity was not determined because of instability. Figure 5B shows the Sephadex elution profile of *V. harveyi* PRAI and InGPS activities. This experiment was performed in the absence of glycerol; although glycerol was generally added to buffers and reaction mixtures to stabilize PRT and TS-B enzyme activities, its presence interferes at the periodate oxidation step of the InGPS assay used. It is clear from Fig. 5B that PRAI and

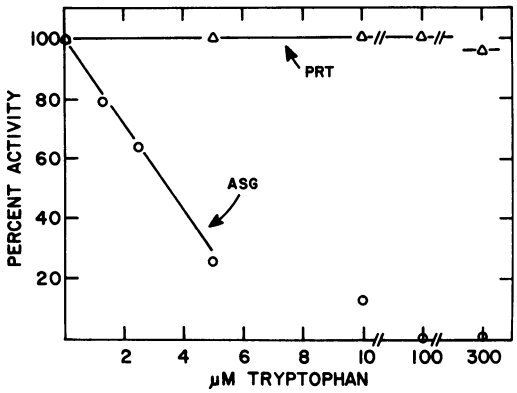

FIG. 4. Feedback inhibition of AS activity by tryptophan. Freshly dissolved L-tryptophan was added to a cuvette, and the reaction was started by adding extract. Since inhibition by tryptophan is opposed by high chorismate levels, exactly 1.0 mM chorismate was used in the assay.

InGPS activities comigrated at a molecular weight of about 45,000, a feature shared by all enteric bacteria examined to date. This suggests that both of these activities are present on a single bifunctional polypeptide.

Partial purification of TS subunits. The TS of bacteria is a tetramer with two different subunits ($\alpha_2\beta_2$). This complex catalyzes the last two steps in the tryptophan biosynthetic pathway, usually at a rate considerably faster than either subunit alone catalyzes its respective half-reaction. In the enteric bacteria the β_2-subunit shows a characteristic heat stability, whereas the α-subunit is stable at low pH values (8); initial experiments on the stability of the two subunits in crude extracts of *V. harveyi* showed a similar pattern of behavior (data not shown).

We attempted a separation and partial purification of the α- and β_2-subunits of TS aimed ultimately at providing separated components for use in enzyme assays for each subunit in the presence of excess complementary subunit, allowing determinations of heat and pH stabilities free from possible interfering agents in crude extracts, studying the state of aggregation of the complex, and possibly testing the enzymic capabilities of intergeneric TS hybrids. Not all of the above goals were reached because of difficulty in stabilizing the α-subunit activity, but one experiment giving partial purification is summarized in Table 5. Crude extract from 45 liters of strain NB5 was briefly heated and treated with protamine sulfate, and the TS activity was found to precipitate at between 35 and 60% ammonium sulfate saturation. After dialysis, the α- and β_2-subunits were separated on a column of DEAE-Sephadex eluted with a salt gradient. A good yield of the β_2-subunit dimer was obtained; this preparation was reasonably free of contaminating material, although not yet pure. The α-subunit eluted in a much broader peak along with the bulk of the cellular protein. Although adequate separation of the two subunits was obtained under these conditions, more work will be needed to stabilize and purify the α-subunit.

In the experiment shown in Table 5, the 35 to

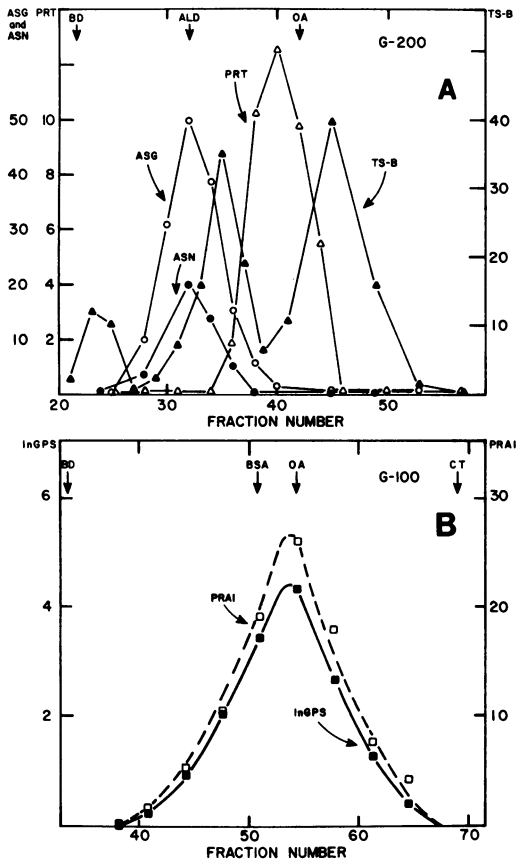

FIG. 5. Estimation of enzyme molecular weights by Sephadex chromatography. (A) An extract of derepressed cells was applied to a column (2 by 50 cm) of Sephadex G-200 and eluted at 4°C with 0.1 M potassium phosphate (pH 7.5) containing 20% glycerol, 50 mM NaCl, 20 mM pyridoxal 5'-phosphate, 10 mM β-mercaptoethanol, 1 mM PMSF, and 0.1 mM EDTA. Four 2.5-ml fractions were collected per h. Elution of [I^{125}]iodide located the bed volume at fraction 67. (B) A column (2 by 30 cm) of Sephadex G-100 was operated as described above but in the absence of glycerol to allow measurement of InGPS activity. The following marker substances were used: blue dextran (BD; excluded volume); aldolase (ALD; molecular weight, 158,000); bovine serum albumin (BSA; molecular weight, 67,000); ovalbumin (OA; molecular weight, 45,000); chymotrypsin (CT; molecular weight, 35,000).

TABLE 5. Partial purification of α- and β-subunits of TS

Stage	Total vol (ml)	Total enzyme units	Total protein (mg)	Sp act	Yield (%)
Crude extract	30	9,120	1,210	7.5	100
Heated supernatant	31	4,680	541	8.5	51
Ammonium sulfate (35–60% saturation)	9.6	6,310	332	19	69
DEAE-Sephadex fractions					
α-Subunit (fractions 35–65)	200	1,020[a]	107.4	9.5	11
β_4-Subunit (fractions 125–155)	200	4,530[b]	103	44	50
TS wash-through (fractions 1–10)	15	810	45	18	9

[a] Assayed with excess β_2-subunit.
[b] Assayed with excess α-subunit.

60% ammonium sulfate fraction containing the presumably multimeric TS aggregate was dialyzed for 18 h with two changes of buffer before chromatography with DEAE column buffer (0.01 M potassium phosphate, pH 7.0, 0.05 M NaCl, 0.02 M pyridoxal 5'-phosphate, 1 mM β-mercaptoethanol, 20% glycerol). The loss of TS activity experienced during dialysis or storage at 4°C was due almost entirely to decay of the α-subunit. In subsequent experiments in which 1 mM EDTA and 0.1 mM PMSF were added to the buffer to protect against proteases, the loss of α-subunit activity was reduced somewhat.

DISCUSSION

Physical properties of the enzymes. In *E. coli* and certain closely related enteric bacteria, the first two reactions of the tryptophan synthetic pathway, AS and PRT, are catalyzed by a tetrameric complex formed from the products of *trpE* and *trpD*, the first two structural genes of the *trp* operon. In this arrangement the *trpD* polypeptide is bifunctional; its proximal one-third furnishes the glutamine amidotransferase function for the AS reaction, and its distal two-thirds catalyzes the PRT reaction (8). In "type 2" enteric bacteria, as well as in most other microorganisms studied, the glutamine amidotransferase function is provided by a separate protein encoded by the *trpG* gene, and there is no physical association of the AS and PRT enzymes. Where an AS-PRT complex occurs, both PRT and AS are subject to feedback inhibition. Largen and Belser (18) studied these two enzymes in 12 enteric bacterial species by using gel filtration and ion-exchange chromatography; five different patterns were observed. These authors found that the physical nature of the first two enzymes of the pathway correlates well with position in the usual taxonomic scheme for the enteric bacteria (18). The tryptophan enzymes of *V. harveyi* exhibit the general characteristics of the enzymes of the *Proteus* and *Erwinia* species studied by Largen and Belser. However, gel filtration of *V. harveyi* AS in crude extracts gave no evidence of a dissociable *trpG* gene product bearing the glutamine binding function, and no *trpG* mutations were found when mutants blocked in the first step of the pathway were examined for both ASN and ASG activities. Therefore, despite the similarity of *V. harvyi* to certain enterobacteria, it would be highly desirable to perform physical studies with purified *V. harveyi* AS to establish the subunit structure of the enzyme.

The heterodisperse gel filtration profile of the TS β-subunit (Fig. 5A) was unexpected. The lability of the α-subunit and the inability to obtain either subunit in pure form forestalled complete analysis of this multimeric enzyme. Successful separation of the subunits on columns of DEAE-Sephadex and the partial purification of the subunits as outlined in Table 5 indicate that such an analysis should be feasible, however. Tris buffer (0.1 M) was found to inactivate TS-B activity in a reaction mixture patterned after the standard reaction mixture for enteric bacteria, but the substitution of phosphate buffer for Tris and the addition of glycerol stabilized the activity for more than 2 h at 37°C. Addition of glycerol was also very useful in stabilizing the PRT activity in crude extract; PRT activity shows a salt dependence, with maximal activity near 0.4 M NaCl, and the isoelectric point of this enzyme as determined by gel focusing is approximately pH 6.6.

Gene expression and regulation. Taken together, the coordinate enzyme expression found in cells growing under a variety of physiological conditions, the existence of the regulatory mutation *trp-1*, which affects all five gene products equally, and transduction mapping data to be presented elsewhere strongly support the hypothesis that the genes responsible for tryptophan biosynthesis in *V. harveyi* are organized in a single operon (*trpPOEDCBA*), an organization possessed only by the *Enterobacteriaceae* among all gram-negative bacteria investigated to date (8). The possible absence of a low-level internal promoter (P2), which is found within

trpD in all of the enteric bacteria that have been studied (17), is at present the only feature not typical of the enteric bacterial *trp* gene organization.

The nature of the *trp-1* mutation in strain NB199 is not entirely clear. This mutation simultaneously elevates the levels of all five tryptophan-synthetic enzymes and is closely linked to the tryptophan structural genes. Such results are expected for either an operator or an attenuator mutation (6), but the possibility of the existence of a repressor locus closely linked to the structural genes has not been ruled out. Thus, although we have shown that strain NB199 and auxotrophs derived from it are capable of severalfold further derepression (Fig. 3 and Table 4), and although *trp-1* maps near the expected operator region, it seems premature to speculate on the nature of this mutation. A promising approach will be to clone the *V. harveyi trp* operon in *E. coli*, as has been accomplished recently for the luciferase genes of this organism (D. H. Cohn, A. J. Mileham, R. C. Ogden, T. O. Baldwin, J. Abelson, M. I. Simon, and K. H. Nealson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, H123, p. 133). Both functional (e.g., the presence or absence of a repressor gene near the operon) and structural analyses could be performed.

A second phenotype of possible regulatory significance is displayed by mutations *trp-40*, *trp-44*, and *trp-46*. When strains with these mutations are subjected to tryptophan starvation, they exhibit activity in all five biosynthetic enzymes (Table 3); however, maximum derepression results in enzyme levels only 10 to 20% of those observed in other auxotrophs. This may be due to impaired promoter function, elevated levels of attentuation, or other aberrations in the regulatory region of the operon.

Taxonomic relationships. In the past, the taxonomy of marine procaryotes has been based on conventional physical and cultural properties, some of which, in light of extensive studies in the past 10 years involving molecular and numerical taxonomy, have proved to be poor indicators of relatedness at or above the generic level (24). It was once suggested that all luminous marine bacteria should be collected in the genus *Lucibacterium*; in *Bergey's Manual*, 8th ed. (12), this genus and the name *Lucibacterium harveyi* are revived. More recently, biochemical, immunological, and nucleic acid hybridization studies have revealed a striking resemblance between common members of the luminous bacterial community and species normally placed in the genus *Vibrio*, especially *Vibrio parahemolyticus*, a cause of acute enteritis (4, 14, 23). Current data favor placing the organism used in our studies, as well as the former *Photobacterium fisheri*, in the genus *Vibrio* as *V. harveyi* and *V. fisheri*, respectively (5). The genus *Photobacterium* (type species, *Photobacterium phosphoreum*) is retained, but the genus *Lucibacterium* is not needed. The family *Vibrionaceae* would then include the genera *Vibrio*, *Aeromonas*, *Plesiomonas*, and *Photobacterium*. Most data, including those reported in this study, point to a close relationship between the *Vibrionaceae* and the *Enterobacteriaceae*. Future investigations should determine whether a single family best portrays the evolutionary relationships in this widespread group of organisms, which includes terrestrial and marine animal, insect, and plant pathogens, as well as free-living forms.

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