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Tryptophanase in Diverse Bacterial Species

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The distribution of tryptophanase was studied. The highest observed specific activity, μmoles per minute per milligram (dry weight) cells, is given in parentheses after each species. Tryptophanase was inducible and repressible in *Escherichia coli* (.914), *Paracolobactrum coliforme* (.210), *Proteus vulgaris* (.146), *Aeromonas liquefaciens* (.030), *Photobacterium harveyi* (.035), *Sphaerophorus varius* (.021), *Bacteroides* sp. (.048), and *Corynebacterium acnes* (.042). The enzyme was constitutive and nonrepressible in *Bacillus alvei* (.013), and was inducible but not repressible by glucose in *Micrococcus aerogenes* (.036). Indole-positive bacteria were found in fecal or intestinal samples from a variety of animals among the mammals, reptiles, insects, molluscs, fish, crustaceans, and amphibians.

Major studies on tryptophanase have utilized *Escherichia coli* as the enzyme source (7, 11). Until recently, tryptophanase was not known to occur in bacteria other than *E. coli*, although indole formation has been observed in many species and is utilized as an identification characteristic. Three additional bacterial species have now been reported to form tryptophanase, *Vibrio cholerae* (1), *Bacillus alvei* (8; R. O. Burns, Ph.D. Thesis, Univ. of Illinois, 1962), and *Pasteurella multocida* (2).

In at least one bacterial species, *B. alvei*, indole excretion may arise by either of two mechanisms (8), i.e., by degradation of indole-glycerol phosphate, an intermediate in tryptophan biosynthesis, catalyzed by tryptophan synthetase, or by degradation of tryptophan, catalyzed by tryptophanase. To establish that indole formation is a consequence of tryptophanase catalysis, it is necessary to demonstrate that indole formation is dependent upon the presence of tryptophan, and to demonstrate the stoichiometric relationships among the products of the reaction, indole, pyruvate, and ammonia.

In this report, we present evidence for the existence of tryptophanase in a variety of different types of bacterial species.

MATERIALS AND METHODS

Organisms and stock cultures. The organisms studied for tryptophanase production were divided into two groups according to method of growth. The first group, composed of aerobic and facultative species, included *Bacillus alvei* strain F (9), *E. coli* T_3 (obtained from C. Yanofsky), *Proteus vulgaris* (ATCC 13315), *Aeromonas liquefaciens* (ATCC 14715), *Photobacterium harveyi* (ATCC 14126), *Paracolobactrum coliforme* (ATCC 11605), and a gram-negative rod isolated from mouse feces. In the second, anaerobic, group were *Micrococcus aerogenes*, obtained from H. R. Whiteley; *Sphaerophorus varius*, ATCC 8501; a species of *Bacteroides* strain B85, obtained from M. P. Bryant; and *Corynebacterium acnes*, ATCC 6919, obtained from J. G. Voss through L. L. Campbell. The designation *Bacteroides* strain B85 is preferred by M. P. Bryant (5). The organism was identified by M. Sebald (Ph.D. Thesis, Institute Pasteur 1962) as *S. funduliformis*, which in turn is stated to be identical to *S. necrophorus* (4).

Aerobes were maintained on Nutrient Agar slants (Difco, lot no. 469396) at 16 C. NaCl (3%) was added to each medium for *P. harveyi*.

Thioglycolate Medium (BBL, lot no. 01-1356) was used for *S. varius* and *Bacteroides*. Except as noted, the cultures (100 ml per 176-ml bottle) were incubated at 37 C and were transferred at 24- to 48-hr intervals.

M. aerogenes was maintained in a medium of the following composition per 100 ml: tryptone, 2.0 g; yeast extract, 1.0 g; glucose, 0.10 g; NaCl, 0.50 g; K_2HPO_4, 0.25 g; sodium pyruvate, 0.40 g; sodium thioglycolate, 0.10 g; and methylene blue, 1 mg. The medium was adjusted to *p*H 7.2 with 10 N KOH and dispensed in 16 by 125 mm screw-cap tubes prior to sterilization. Immediately after inoculation, the tubes were stored at −20 C. The culture was placed at 37 C 24 hr before use.

C. acnes was maintained at 16 C on Brain Heart Infusion Agar (Difco lot no. 475178) containing 1.0% glucose.

Growth and sampling. Starter cultures, 10 ml per 50-ml Erlenmeyer flask, of the aerobes were obtained by growing the organism in Nutrient Broth and then transferring twice at 24-hr intervals through the same medium to be used in the particular experiment. The cultures were grown on a rotary shaker at 37 C, except for *A. liquefaciens* and *P. harveyi*, which were

TABLE 1. *Media*[a]

1. Standard[b]
2. Standard + 1% glucose
3. Standard + 500 μg of L-tryptophan per ml
4. Standard + 1% glucose + 500 μg of L-tryptophan per ml
5. Trypticase (2%; BBL, lot no. 705688)
6. Tryptone (2%; Difco, lot no. 485051)
7. Tryptone (2%) + 1% glucose
8. Tryptone (2%) + 0.5% yeast extract (Difco, lot no. 494288)

[a] *P. harveyi* was grown with 3% NaCl added to all media. Media for *B. alvei* contained 1 g of thiamine·HCl per ml. For *E. coli* strain T_3, 1 μg of tryptophan per ml was added to media 1 and 2. All media for *S. varius* and *Bacteriodes* contained the following additives per 100 ml: yeast extract, 0.50 g; glucose, 0.10 g; and sodium thioglycolate, 0.10 g. Media 5–8 also included 2.5 ml of a potassium phosphate solution (200 g of K_2HPO_4 and 45 g of KH_2PO_4 per liter) per 100 ml of medium. All media for *M. aerogenes* contained (per 100 ml of medium): yeast extract, 0.5 g; sodium pyruvate, 0.40 g; and sodium thioglycolate, 0.10 g. The media for *C. acnes* contained (per 100 ml) 0.2 g of glucose and 0.1 g of sodium thioglycolate; in addition, media 1–4 contained 0.5 g of yeast extract; and media 5–8 contained 0.5 g of NaCl and 0.25 g of K_2HPO_4.

[b] Standard medium of Beozi and DeMoss (3) contains (per 100 ml): 0.1 g of NH_4Cl, 0.6 g of Na_2HPO_4, 0.3 g of KH_2PO_4, 0.5 g of NaCl, 0.01 g of $MgSO_4$, and 1.0 g of Vitamin Free Casamino Acids (Difco, lot no. 489496).

grown at 30 C. Starter cultures for the anaerobes were obtained by transferring the organism (5-ml inoculum) three times through the medium to be used in the experiment. The cultures, 100 ml per 176-nl bottle, were grown at 37 C without mixing.

For growth and enzyme production studies, the organisms were grown on eight media at both 25 and 37 C (Table 1).

Aerobic cultures, 100 ml per 500-ml Erlenmeyer flask, were inoculated to an initial A_{660} of 0.01 and grown in a shaker water bath. The anaerobic cultures, 100 ml per 176-ml screw-cap bottle, were inoculated to an initial A_{660} of 0.04, and grown without shaking. Approximate equivalence of A_{660} and dry weight are given in Table 2.

Samples for the determination of indole, dry weight of cells, and tryptophanase activity were taken at 16, 24, and 42 hr. The anaerobic cultures were mixed by inversion prior to sampling. For *Bacteroides*, since mixing at sample time usually caused decreased subsequent rates of growth, six cultures for each medium and temperature were prepared. Cultures 1 and 2 were sampled at 16 hr, 1–4 at 24 hr, and 1–6 at 42 hr.

To obtain cells for the preparation of crude extracts, cultures (1 liter per 2,800-ml Fernbach flask) of the aerobes were grown on a rotary shaker at 37 or 30 C. *E. coli*, *P. coliforme*, the mouse isolate, and *B. alvei* were grown on medium no. 5 at 37 C. For *B. alvei*, 1.0 g of glucose and 10 μg of thiamine hydrochloride were added per 100 ml of medium. *A. liquefaciens* and *P. harveyi* were grown at 30 C on medium no. 6. The medium for *P. harveyi* contained 3.0 g of NaCl and 100 μg of tryptophan per 100 ml. *P. vulgaris* was grown at 37 C on medium no. 6.

TABLE 2. *Occurrence of tryptophanase in a variety of bacterial species*

Organism	Cell weight[a]	Ind[b]	Rep[c]	Stoichiometry[d]			Specific activity[e]
				Indole	Pyruvate	NH_2	
Bacillus alvei F	.46	−	−	1.00	0.99	1.04	0.013
Paracolobactrum coliforme 11605	.28	+	+	1.00	0.97	1.01	0.210[f]
Escherichia coli T_3		+	+	1.00	1.01	0.99	0.914
Proteus vulgaris 13315	.39	+	+	1.00	1.02	1.21	0.146[f]
Aeromonas liquefaciens 14715	.42	+	+	1.00	0.99	1.11	0.030
Photobacterium harveyi 14126	.43	+	+	1.00	0.96	0.96	0.035[f]
Micrococcus aerogenes	.39	+	−	1.00	1.08	1.37	0.036
Sphaerophorus varius 8501	.59	+	+	1.00	1.01	—[g]	0.021
Bacteroides sp.	.69	+	+	1.00	0.99	1.26	0.048
Corynebacterium acnes 6919	.37	+	+	1.00	0.98	0.99	0.042
Mouse isolate	.28	+	+	1.00	1.00	1.02	0.145[f]

[a] Cell weight is expressed as milligrams of cells (dry weight) per unit of absorbancy at 660 nm.
[b] Inducibility.
[c] Repressibility by glucose.
[d] Relative amounts of products, normalized to indole.
[e] Expressed as μmoles of indole formed per minute per milligram of cells (dry weight); cells were grown at 37 C.
[f] Cells were grown at 25 C.
[g] Ammonia was not measurable because of the formation of a colloidal suspension.

Cells were harvested at 16 to 24 hr by centrifugation at 6,000 × *g* for 30 min. The cells were stored at −20 C.

The anaerobes were grown in a 14-liter fermentor (New Brunswick Scientific Co., New Brunswick, N.J.; type F-14) at 37 C. *M. aerogenes* was grown under N_2 on medium no. 6 with the indicated additives. *Bacteroides* was grown on medium no. 5 with 0.5 g of yeast extract, 0.1 g of sodium thioglycolate, 1.0 g of glucose, and 500 μg of tryptophan per 100 ml. *S. varius* was grown on medium 5 with 0.5 g of yeast extract, 1.0 g of glucose, 0.1 g of sodium thioglycolate, and 50 mg of tryptophan per 100 ml. The cultures of *S. varius* and *Bacteroides* were gently aerated with N_2 only until growth began, as estimated visually. The cells were harvested in a Sharples supercentrifuge and were stored at −20 C.

Preparation of whole-cell suspensions. Whole-cell suspensions were prepared by sedimenting the cells from 5 ml of culture at 8,700 × *g* for 15 min. The cells were washed and suspended in 5 ml of 0.05 M potassium phosphate buffer (*p*H 7.2). Cell density was determined turbidimetrically by means of a Gilford Model 300 spectrophotometer. The absorbancy readings at 490 nm were converted to milligrams (dry weight) of cells by the use of a previously determined conversion factor. All solutions for *P. harveyi* contained 3% NaCl.

Preparation of crude extracts. Frozen cells were thawed and suspended in four volumes [grams (wet weight) per milliliter] of 0.05 M potassium phosphate (*p*H 7.2) containing 10^{-4} M pyridoxal-5′-phosphate. The suspended cells were subjected to five 1-min treatments with a Branson Sonifier. Except for *Bacteriodes* and *Sphaerophorus*, the disrupted cell suspension was centrifuged at 30,000 × *g* for 20 min and the pellet was discarded. *Bacteroides* and *S. varius* were centrifuged for 30 min at 60,000 × *g*. The resulting supernatant fluids were dialyzed (two changes) overnight at 4 C in 30 volumes of 0.05 M potassium phosphate (*p*H 7.2) containing 10^{-4} M pyridoxal-5′-phosphate. The buffer used for suspension of *P. harveyi* cells contained 3% NaCl.

Indole determination. Indole was determined by a modification of the method of Yanofsky (13). *A. liquefaciens*, *P. vulgaris*, and the rat isolate all formed pigments ranging from brown to red in color. The pigments interfered with the indole determination. In these cases, samples were extracted with an equal volume of toluene, and 0.5 ml of the toluene layer, or an appropriate dilution thereof, was mixed with 3 ml of color reagent (948 ml of 95% ethyl alcohol, 52 ml of 36 N H_2SO_4, and 14.7 g of *p*-dimethylaminobenzaldehyde) and allowed to stand at room temperature for 20 min. For all other species, 0.5 ml of the culture, or of an appropriate dilution, was mixed with 3 ml of color reagent and centrifuged at 2,400 × *g* during the 20-min incubation period to remove insoluble cell debris. The absorbancy was measured at 568 nm.

Tryptophanase activity. Tryptophanase activity was determined by a modification of the method of Pardee and Prestidge (12). A sample, 0.1 ml, of cell suspension was mixed with 0.2 ml of the complete reaction mixture plus 4 drops of toluene (from a disposable Pasteur pipette) and incubated at 37 C for 5 min. The complete reaction mixture was prepared by adding to 100 ml of 0.05 M potassium phosphate (*p*H 7.5): 2.75 mg of pyridoxal-5′-phosphate, 19.6 mg of disodium ethylenediaminetetraacetatedihydrate, 10 mg of bovine serum albumin, and 87.5 ml of distilled water. To start the reaction, 0.2 ml of .02 M L-tryptophan was added and the incubation was continued for 10 min. The reaction was terminated by the addition of 3 ml of color reagent, and indole was estimated as described above. The solutions for *P. harveyi* contained 3% NaCl.

One unit of enzyme is defined as that quantity which will produce 1 μmole of indole per min under the assay conditions. Specific activity is expressed as units per milligram (dry weight) of cells.

Reaction for determination of all end products. When all end products were to be assayed, a reaction mixture containing 4 ml of complete reaction mixture, 4 ml of .02 M L-tryptophan, and 2 ml of crude extract (or an appropriate dilution) was incubated at 37 C for 20 min. The reaction was terminated by the addition of trichloroacetic acid to a final concentration of 10%. After 15 min at 0 C, the precipitated protein was removed by centrifugation at 8,700 × *g* for 15 min. The supernatant fluid was assayed for indole, pyruvate, and ammonia.

Pyruvate. Pyruvate was determined by the method of Friedemann and Haugen (6). Crystalline sodium pyruvate was used as the standard.

Ammonia. A 1- to 2-ml amount of the deproteinized supernatant was placed in the outer well of a Conway diffusion dish, and the ammonia was released by the addition of 1 ml of saturated Na_2CO_3. The inner well contained 1 ml of 0.02 N H_2SO_4. After 12 hr at room temperature, the ammonia was determined by a modification of the method of Johnson (10). A sample of the inner well was brought to a volume of 3 ml by adding water; 2 ml of color reagent and 0.6 ml of 10 N NaOH were added subsequently. After allowing the sample to stand for 15 min at room temperature, we determined the absorbancy at 410 nm. Crystalline $(NH_4)_2SO_4$ was used as the standard.

RESULTS AND DISCUSSION

The cultures were grown in the media and under the conditions described in Materials and Methods. Culture samples were taken at 16, 24, and 42 hr and analyzed for growth (A_{660}) and indole production. Samples were centrifuged, the sedimented cells were washed and suspended, and the ability to degrade L-tryptophan to indole was tested (Table 2). The enzyme was considered to be inducible if the specific activity was at least threefold higher when the organism was grown in the presence of tryptophan than in the same medium in the absence of tryptophan. Enzyme synthesis was considered to be repressible if the specific activity of cells grown under inducing conditions was decreased by at least 70% when the medium contained 1% glucose. In the experi-

ments with *S. varius*, *Bacteroides*, and *C. acnes*, when 0.1 to 0.2% glucose was present in the "basal" medium, the effect of an additional 1% glucose was judged to be repression.

Specific activity determinations were done with fresh whole-cell suspensions and are based on cell dry weight. Toluene was added to the reaction mixtures to destroy the permeability barrier. The specific activity value given represents the highest value observed for the particular species under any of the growth conditions. The specific activity values of different species were not necessarily highest at the same temperature, or in the same medium, or at the same sample time.

Stoichiometry was determined with dialyzed, freshly prepared, extracts. The values given are normalized to the amounts of indole formed, and are the mean values of from 3 to 5 complete analyses for each species.

It is clear from the results in Table 2 that tryptophanase is formed by bacterial species which are physiologically and morphologically diverse. The single common attribute of all tryptophanase-synthesizers, with the possible exception of *P. harveyi*, appears to be the ability to grow in an animal intestinal tract. This observation was suggested to the senior author by a participant in a Summer School for Microbiology held at Uttar Pradesh Agricultural University, Pant Nagar, India, in 1965. As an approach to the study of the metabolic function of tryptophanase, we have used the working hypothesis that the ability to form tryptophanase is related to the ability of certain bacterial species to survive successfully in an animal intestinal tract. A corollary to the hypothesis is the prediction that in every animal intestinal tract, one may expect to find at least one bacterial species which can form tryptophanase.

The corollary has been tested. Table 3 presents data obtained in a survey of a variety of animals. Usually fecal samples were used, but in some cases gut contents were sampled after the animals were killed. Virtually all of the animals surveyed yielded organisms that are capable of forming indole during growth on 2% tryptone.

Diluted samples were spread on Nutrient Agar plates and incubated aerobically overnight at 37 C. Isolated colonies were transferred to 1-ml volumes of 2% tryptone and incubated at 37 C for 24 hr. Each culture was assessed for indole formation by adding 3 ml of color reagent directly to the culture. The results are given as the percentage of total cultures able to form indole. No attempt was made to identify any of the cultures. It is possible that in some cases, perhaps all, the indole-positive cultures were ultimately of human source, a result of the proximity of the animals to humans; i.e., some animals were captive, some were domestic, and some were derived from habitats, e.g., streams, which possibly were polluted by human excrement. Regardless of the ultimate source of the bacteria, the results are consistent with the hypothesis that the ability to form tryptophanase is related to the ability of certain bacteria to survive successfully in an animal intestinal tract.

TABLE 3. *Occurrence of indole-positive bacteria in a variety of animal species*

Source	Colonies	
	Total	Indole-positive
		%
Snake (*Boa constrictor*)	200	48.0
Snake (not identified)	200	100.0
Tomato worm (*Phlegethontius sexta*)	199	0.0
Lizard (*Gekko gekko*)	199	0.0
Salamander (*Ambystroma mexicanum*)	249	13.3
Guinea pig (*Cavia porcellus*)	200	2.5
Dog (*Canis familiaris*)	94	83.0
Sheep (*Ovis aries*)	212	9.2
Swine (*Sus scrofa*)	205	64.9
Horse (*Equuis caballus*)	200	0.0
Goat (*Capra hircus*)	198	76.2
Snail (not identified)	202	38.1
Pickerel (*Esox vermiculatus*)	201	58.7
Crayfish (*Cambarus affinis*)	200	28.5
Frog (*Rana pipiens*)	200	71.0
Man (*Homo sapiens*)	65	27.7

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