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Effect of D-Tryptophan on Hemolysin Production in *Vibrio parahaemolyticus*

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Production of the Kanagawa hemolysin by a strain of *Vibrio parahaemolyticus* isolated from a gastroenteritis patient was found to correlate with the presence in cell lysates of two unidentified compounds, designated X and Y. The two compounds were present in cell lysates of the organism grown in peptone at the optimal pH for hemolysin synthesis but were not present when cell lysates were grown in peptone at a constant pH of 8.0. They were also absent in cells grown in synthetic medium at pH 6.2 without the addition of D-tryptophan, a condition under which hemolysin is not produced. Both X and Y were present intracellularly only from the time that D-tryptophan was added to synthetic medium, a known method of inducing hemolysin synthesis.

When *Vibrio parahaemolyticus* was grown in a defined salts medium at pH 6.2, which is the optimal pH for hemolysin production in peptone-based media, the amount of hemolysin produced was negligible unless 0.5 mM D-tryptophan was added to early-logarithmic-phase cultures (2). After D-tryptophan was added, growth, as well as carbon and nitrogen substrate utilization, were inhibited for 7 to 12 h depending on the cell density at the time of tryptophan addition. During this time, hemolysin could be detected only in cell lysates. The toxin appeared in the supernatant only when growth recommenced after the period of growth inhibition.

It was unknown whether D-tryptophan actually entered the cell, why growth resumed if D-tryptophan was indeed toxic, whether hemolysin production depended on the constant presence of D-tryptophan in the cell, or whether the metabolites of D-tryptophan induced hemolysin production. This paper reports a high-pressure liquid-chromotographic (HPLC) examination of the levels of D-tryptophan and other compounds in a culture of *V. parahaemolyticus* and the relationship of these levels to the production of hemolysin.

MATERIALS AND METHODS

Culture examined. *V. parahaemolyticus* NCTC 10886, isolated by G. I. Barrow from a case of food poisoning acquired in the Far East, was obtained from the National Collection of Type Cultures. The strain was Kanagawa positive (hemolytic) when tested on Wagatsuma blood agar (4).

Culture media. The complex medium used contained 2.0% peptone (Difco Laboratories, Detroit, Mich.) and 3.0% NaCl (BDH; reagent grade, product number 30123) in 0.03 M KH_2PO_4–K_2HPO_4 (pH 8.0) sterilized by autoclaving. The basal synthetic medium (BSM) contained 3.0% NaCl, 2.0% KH_2PO_4, 0.25% $(NH_4)_2SO_4$, 0.2% mannitol, and 0.025% $MgSO_4 \cdot 7H_2O$ adjusted to pH 7.0 with KOH and sterilized through a 0.45-μm filter (Millipore Corp., Bedford, Mass.). The inducing medium (BSM-M) had an increased carbon source (0.8% mannitol) and was adjusted to pH 6.2 with KOH before filtration. D-Tryptophan was dissolved in 3% NaCl to 25 mM and then added to the culture as required.

Culture conditions. *V. parahaemolyticus* NCTC 10886 was subcultured for at least three 8-h periods in 50 ml of peptone broth (pH 8.0, at 37°C), with rotary shaking at 250 rpm in a 1-in. (2.54-cm) orbit. The last peptone subculture was harvested after 2 to 4 h of growth (optical density at 600 nm [OD_{600}], 0.1 to 0.4), washed in sterile 3.0% NaCl, and then diluted in synthetic medium (BSM, pH 7.0) to 3.0×10^6 cells per ml. When this culture was in logarithmic growth (OD_{600}, 0.1) after 8 to 12 h of incubation with shaking at 37°C, the cells were again harvested, washed in 3.0% NaCl, and diluted in 600 ml of fresh salts medium (BSM-M, pH 6.2) in a 2,800-ml Fernbach flask. The culture was then grown under pH-stat conditions (1). When the culture reached an OD_{600} of 0.3, 5 h after inoculation, D-tryptophan was added to a final concentration of 0.5 mM.

Determination of growth. Growth in the fluid medium was determined turbidimetrically at 600 nm by using a Unicam SP 1800 spectrophotometer (Pye Instruments, Cambridge, England).

Determination of hemolytic activity. Hemolytic activity was determined as previously described (2).

Cell lysis. Samples were taken aseptically from the culture (120 ml for 5 h postinoculation; 60 ml for 5.5, 9, and 13 h postinoculation; and 30 ml for 17 and 25 h postinoculation) and centrifuged at $10,000 \times g$ for 10 min at 22°C, and the supernatant was removed for further study. The cells were then washed once in 1% NaCl, suspended in 3 ml of distilled water, and frozen at −20°C. A single freeze-thaw cycle was followed by four 15-s durations at maximum tuning with a BP-2 ultrasonic disintegrator (Blackstone Ultrasonics, Inc., Sheffield, Pa.). Lysis was complete as judged by microscopic examination.

HPLC of aromatic compounds. Waters Associates (Milford, Mass.) delivery systems (models 6000A and M-4S) were used in conjunction with a universal liquid-chromatography injector (model U6K), solvent programmer (model 660), absorbance detector (model 440), and data module (model 730). Samples were prepared by placing 0.4 ml of culture supernatant, cell lysate, or chemical standards in a Brinkmann Instruments, Inc. (Westbury, N.Y.) micro test tube, followed by 0.4 ml of 0.5 M trichloroacetic acid and 0.2 ml of 1.0 M $KHCO_3$ with mixing after each addition. The

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TABLE 1. HPLC of intracellular and extracellular aromatic compounds when *V. parahaemolyticus* NCTC 10886 is grown in a salts medium

Time after inoculation (h)	Relative peak height[a] of compound in:						HU/ml[c]
	Culture supernatant			Cell lysate			
	D-TRP	X	Y	D-TRP	X	Y	
5	0	0	0	0	0	0	0 (0)
5.5[b]	100	0	0	860	10	7	0 (0)
9	102	0	0	1,200	9	7	2.2 (0)
13	98	3	1	900	6	12	4.5 (0)
17	100	1	0	0	23	30	7.0 (3.0)
25	102	1	0	0	100	100	14.1 (13.5)

[a] For calculating the relative peak height for D-tryptophan (D-TRP) in culture supernatants, 0.5 mM amino acid was set equal to 100. D-Tryptophan in cell lysates was calculated from the volume (cm^3) of cells used for HPLC studies. For calculating the relative peak heights of X and Y, the unknown compounds (see text), 100 was set equal to the peak height of each at 25 h of incubation of the culture. The retention times on HPLC were 15.6 min for D-tryptophan, 6.3 min for compound X, and 9.9 min for compound Y.

[b] 0.5 mM D-tryptophan added.

[c] HU/ml, Hemolytic units per milliliter for lysate (hemolytic units per milliliter in supernatant are in paretheses). Growth started again after 16 h when hemolysin was released into the medium.

neutralized solution was spun in an Eppendorf model 5412 centrifuge (2 min at 15,600 × *g*) to remove insolubles. Samples of 0.01 ml of the resulting supernatant were separated on a C18 μBondapak column (Waters Associates) at 1.0 ml per min with a 12-min linear gradient of 0 to 25% methanol in 0.1 M potassium phosphate buffer, pH 6.0. This was followed by isocratic high-buffer elution. The absorbance was monitored at 280 nm.

RESULTS AND DISCUSSION

Culture supernatants. The synthetic medium (BSM-M) contained no polypeptides or aromatics as a carbon source, and no material absorbing at 280 nm could be detected in culture supernatants of growing cells. After the addition of 0.5 mM D-tryptophan, only this amino acid was evident in culture supernatants for the duration of the incubation (i.e., to 25 h after inoculation). Neither peak height (0.087 for absorbance at 280 nm) nor retention time (15.6 min) for D-tryptophan varied on the HPLC readings; therefore, metabolism of this compound was negligible. Thus, the recovery of growth and substrate utilization of *V. parahaemolyticus* after the addition of D-tryptophan could not be attributed to the degradation of this amino acid (see Table 1).

D-Tryptophan induction of hemolysin synthesis in synthetic media is a balance between the biomass formed at the time of addition and the concentration of D-tryptophan needed to cause growth inhibition. Hence the period of inhibition will increase if the D-tryptophan concentration is increased. The growth rate of *V. parahaemolyticus* is rapid with a generation time of 35 min in synthetic medium (2); thus, it is important not to miscalculate the timing of D-tryptophan addition. If 0.5 mM D-tryptophan is added when the OD_{600} is above 0.4, the growth will not be inhibited and no hemolysin will be induced. This can be avoided by increasing the amount of D-tryptophan. If the OD_{600} is less than 0.3, the 0.5 mM D-tryptophan will cause growth inhibition, but there will be too few cells to reliably analyze. Despite this delicate balance, the method is highly reproducible.

Cell lysates. A correlation was found between the presence of D-tryptophan in the cell and the inhibition of culture growth and substrate utilization. During the time of growth inhibition, D-tryptophan accumulated in cells and disappeared when the culture resumed growth and substrate utilization. Therefore, D-tryptophan in the cell correlated with growth inhibition. It has been reported that D-tryptophan inhibits beta-galactosidase synthesis in *Escherichia coli* (3). This amino acid isomer may inhibit *V. parahaemolyticus* in a similar manner.

Hemolysin production continued for the duration of the incubation or 12 h after the time when D-tryptophan was no longer detectable in the cell (2). Two unidentifiable compounds, designated X and Y, were present during this production and within 0.5 h of D-tryptophan addition (see Table 1 and Fig. 1). Standards showed that these compounds were neither metabolic products of D-tryptophan (D-kynurenine, kynurenic acid) nor of L-tryptophan (indole, L-kynurenine, anthranilic acid, catechol), although it was known that 100 μM L-kynurenine or 100 μM anthranilic acid acted as inducers of hemolysin production in synthetic media. Additional studies have shown that X and Y appeared in the cell whenever hemolysin was synthesized.

Experiments were repeated with *V. parahaemolyticus* grown under pH-stat conditions in 2.0% peptone with or without 0.5% mannitol. Under these conditions hemolysin is maximally produced, but when experiments were conducted at pH 8.0, the amount of hemolysin formed was at the limit

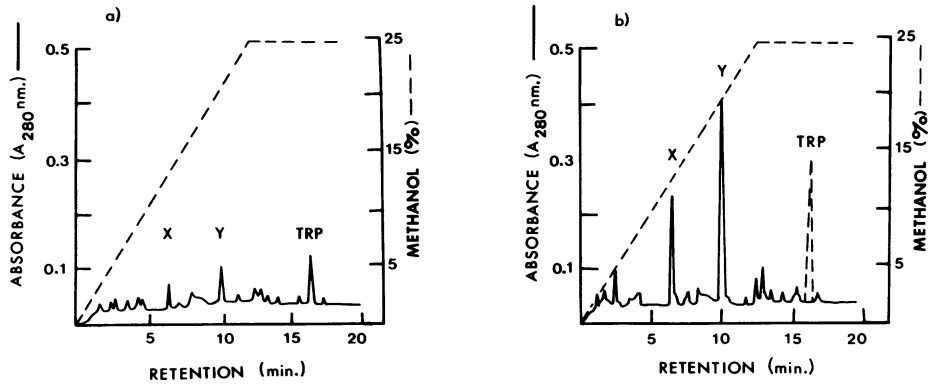

FIG. 1. HPLC analysis of cell lysates from *V. parahaemolyticus* NCTC 10886 grown in synthetic medium with 0.5 mM D-tryptophan. (a) Intracellular sample of cells 5.5 h after culture inoculation (0.5 h after D-tryptophan induction). X and Y, two unknown compounds (see text); TRP, D-tryptophan. (b) Intracellular sample of cells 25 h after culture inoculation (20 h after the addition of D-tryptophan). TRP, Location of D-tryptophan had it been present in the cell lysate.

of detection in the quantitative assay (1). Cells taken from these cultures were lysed, and the intracellular contents were examined by HPLC. Only the hemolytic cultures contained detectable X and Y. Mass-spectrometric analysis of X and Y has shown that they are high-molecular-weight heterocyclics (K. Laderoute, personal communication), but their identities can not be determined by this method. In light of their significance in hemolysin production, the characterization of these two compounds is currently under way.

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