Production of the Kanagawa Hemolysin by *Vibrio parahaemolyticus* in a Synthetic Medium

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A synthetic medium capable of supporting growth of *Vibrio parahaemolyticus* is described. Growth yields and generation times were comparable to growth in a complex medium, although Kanagawa hemolysin was undetectable in the synthetic medium. Upon the addition of single amino acids to this synthetic medium, only D-tryptophan induced production of the hemolysin. L-Tryptophan was found to inhibit the action of the D-stereoisomer. The response to D-tryptophan was pH dependent; the greatest hemolysin expression occurred at pH values below 6.5. The addition of 100 μM D-tryptophan to early-logarithmic-phase cultures caused an inhibition of growth and of substrate utilization, both of which lasted 7 h. During this time, hemolysin was produced only intracellularly. The hemolysin appeared in the supernatant only when growth recommenced. The hemolysins within the cell and in the supernatant were both inactivated by antisera raised against the standard Kanagawa hemolysin.

Chemically defined media have been reported which sustain growth of *Vibrio parahaemolyticus* over a few generations (4, 6). However, as yet there has been no report of a synthetic medium in which the organism can grow upon repeated subculture. Such a medium is necessary in any investigation of the mechanism of hemolysin production, and a defined salt medium has been formulated to meet these requirements. As previously reported (2), the carbohydrate source has little influence on the production of the Kanagawa hemolysin by *V. parahaemolyticus* in complex medium. In this report we have investigated the role of amino acids on hemolysin production in a synthetic medium.

MATERIALS AND METHODS

Cultures 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 (NCTC), Central Public Health Laboratory, Colindale, London, U.K. Indole-negative strains that were hemolytic were kindly supplied by M. Ohashi of the Tokyo Metropolitan Research Laboratory of Public Health, Tokyo, Japan.

Culture media. The complex medium used was 2.0% peptone (Difco Laboratories) and 3.0% NaCl (BDH reagent grade, product number 30123) in 0.03 M KH₂PO₄-K₂HPO₄ (pH 8.0) sterilized by autoclaving. The basal synthetic medium (BSM) was 3.0% NaCl, 2.0% KH₂PO₄, 0.25% (NH₄)₂SO₄, 0.2% mannitol, 0.025% MgSO₄·7H₂O adjusted to pH 6.2 with KOH and sterilized through 0.45-μm filters (Millipore Corp., Bedford, Mass.). Single amino acids were added before inoculation by first dissolving 25 mM of each amino acid in 3% NaCl (except for tyrosine, which was dissolved in 3% NaCl containing 0.1 M HCl), sterilizing by filtration, and then adding each amino acid as required to 50 ml of medium in a 250-ml flask. All chemicals other than the NaCl were of analytical grade.

Culture conditions. *V. parahaemolyticus* NCTC 10886 was subcultured for at least three 8-h subcultures in 50 ml of peptone broth (pH 8.0 at 37°C), with shaking at 250 rpm. The last peptone subculture was harvested after 2 to 4 h of growth (optical density at 600 nm [OD₆₀₀] of 0.1 to 0.4), washed in sterile 3.0% NaCl, and then diluted in synthetic medium (pH 7.0) to 3.0 × 10⁶ cells per ml. When this culture was in logarithmic growth (OD₆₀₀ of 0.1) after 12 h of incubation at 37°C, the cells were again harvested, washed in 3.0% NaCl, and diluted in fresh salt medium to 3.0 × 10⁶ cells per ml. When prolonged sampling was necessary, volumes were increased to 600 ml in 2,800-ml Fernbach flasks. Details of the pH-stat methods are published elsewhere (2).

Determination of growth. Growth in the fluid medium was determined turbidimetrically at 600 nm by using a Unicam SP 1800 spectrophotometer (Pye Instruments, Cambridge, Mass.), by viable counts on 2.0% peptone (Difco), 3.0% NaCl, and 0.5% K₂HPO₄ supplemented with 1.5% Difco agar, or by dry weight by filtering the culture through tared 0.45-μm membranes (Millipore Corp.) and drying at 95°C for 12 h. Protein was determined by the method of Lowry et al. (5); RNA was determined by the Mejbaum orcinol method (8).

Determination of hemolytic activity. Hemolytic activity was determined essentially as previously described (2), except that the hemolysin preparation was not heat shocked, and the buffer was 0.9% NaCl-5 mM CaCl₂·2H₂O-0.01 M Tris-hydrochloride (pH 7.0).
RESULTS AND DISCUSSION

Growth in synthetic media. V. parahaemolyticus NCTC 10886 grew poorly in the medium of Morishita and Takeda (6) with a generation time of 65 min. When the phosphate concentration was raised from 0.02 to 0.15 M, however, the generation time decreased to 35 min. Subculturing in this latter medium every 8 h with a 0.2% (vol/vol) inoculum showed little variation either in growth rates or yields over eight subcultures. Table 1 compares the growth in peptone and synthetic media.

Effect of amino acids. V. parahaemolyticus NCTC 10886, grown in BSM under a controlled pH 6.2, did not produce hemolysin. When single amino acids were added at concentrations between 0.1 and 1.0 mM to the BSM, the cultures had comparable growth over a 24-h period, although L-cysteine and all the D-amino acids gave a temporary inhibition of growth which lasted for 6 to 12 h. The L-amino acids tested were alanine, arginine, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine; the DL-amino acids tested were alanine, aspartic acid, isoleucine, phenylalanine, serine, threonine, tryptophan, tyrosine, and valine; D-amino acids tested were leucine, serine, and tryptophan. Only in the case of D-tryptophan was hemolysin produced. Substituting glucose for mannitol or nitrate for ammonium in BSM did not alter these results. It seems unlikely, therefore, that classical catabolite repression (7) played a major role in the failure of the other amino acids to induce hemolysin synthesis.

Both L-tryptophan and DL-tryptophan gave cultures a hemolytic activity that was at the limit of sensitivity for the hemolysin assay (0.1 hemolytic unit per ml), whereas D-tryptophan gave 4.6 hemolytic units per ml at the same pH of 6.2. As the latter compound is found within the DL form, it was puzzling that DL-tryptophan proved ineffective in stimulating hemolysin production. Subsequent studies showed that L-tryptophan inhibits the hemolysin inducing effect of D-tryptophan, yielding little expression of the hemolysin.

TABLE 1. Comparison of growth of V. parahaemolyticus NCTC 10886 in complex and defined media

<table>
<thead>
<tr>
<th>Medium</th>
<th>Cell yield after 16 h</th>
<th>Generation time (min)</th>
<th>Viable count (CFU/ml)</th>
<th>Dry wt (mg/ml)</th>
<th>OD&lt;sub&gt;600&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peptone&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13</td>
<td>6.4 ± 1.2 × 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>5</td>
<td>1.30</td>
<td></td>
</tr>
<tr>
<td>BSM&lt;sup&gt;b&lt;/sup&gt;</td>
<td>35</td>
<td>3.0 ± 0.3 × 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>2</td>
<td>0.75</td>
<td></td>
</tr>
<tr>
<td>BSM-M&lt;sup&gt;c&lt;/sup&gt;</td>
<td>35</td>
<td>1.3 ± 0.2 × 10&lt;sup&gt;9&lt;/sup&gt;</td>
<td>6</td>
<td>2.60</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> CFU, Colony-forming units.
<sup>b</sup> See the text.
<sup>c</sup> As BSM but with 0.8% mannitol instead of 0.2% mannitol.
neutrality, the hemolysin production increases, reaching maximal when the pH drops below 6.5. This response was repeated when *V. parahaemolyticus* was grown in BSM supplemented with 0.1 mM D-tryptophan (Fig. 2). This inducer was relatively ineffective in medium with alkaline pH, but gave high expression when the pH was below 6.5. As the medium was strongly buffered with 0.15 M KH₂PO₄-K₂HPO₄ buffer and had a low carbon source (0.2% mannitol), the final pH of the cultures varied little from the initial pH. Final growth yields as measured by OD₆₀₀ were also comparable.

Antiserum raised against the purified Kanagawa hemolysin produced in peptone medium inactivated the D-tryptophan–induced hemolysin produced in BSM. Figure 3 shows that these hemolysins and an authentic preparation of Kanagawa hemolysin provided by Y. Miyamoto have a "reaction of identity."

**Effect of D-tryptophan.** To study the growth effects of D-tryptophan, it was added to a final concentration of 0.5 mM to a logarithmic-phase culture in BSM-M (BSM containing 0.8% mannitol). Although D-tryptophan had an immediate effect on the growth of the culture, total inhibition of growth did not occur until 3 h after its addition, and then for only 7 h (Fig. 4a). After this time, the culture reestablished growth. Utilization of mannitol and ammonium also followed

Comparison of hemolysins. In a previous publication (2) we have reported that the expression of the Kanagawa hemolysin is affected by the pH. In complex medium, as the pH drops below

FIG. 3. Ouchterlony plate of purified Kanagawa hemolysins and anti-Kanagawa antiserum. (A) Hemolysin purified from *V. parahaemolyticus* NCTC 10886 grown in complex medium; (B) Kanagawa hemolysin provided by Y. Miyamoto; (C) hemolysin partially purified from *V. parahaemolyticus* NCTC 10886 grown in synthetic medium with 100 µM D-tryptophan; (D) antiserum provided by Y. Miyamoto to his Kanagawa hemolysin.
Tryptophan metabolites

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Effect of TABLE

Tryptophan
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was
minor,
the
hemolysin
hemolysin
were
greater
carbon
source
that
gested
ysin
were
inducers,
possible
the
cell
was
production
by
L-tryptophan,
that
were
acids,
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but
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is
unknown.

Therefore, it was considered that L-tryptophan is not an effective inducer for V. parahaemolyticus NCTC 10886 because of the active degradation of L-tryptophan by tryptophanase. However, upon testing indole-negative strains for L-tryptophan induction no hemolysin was produced. Investigations are currently under way to determine whether the stimulation of hemolysin by D-tryptophan is induction in the classical sense, or whether it is derepression through a metabolic imbalance in benzenoid synthesis in cells of pathogenic strains of V. parahaemolyticus.

LITERATURE CITED


TABLE 2. Effect of tryptophan-related compounds on hemolysin production by V. parahaemolyticus NCTC 10886

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (µM)</th>
<th>Growth (OD₆₅₀)</th>
<th>Hemolytic activity (H.U./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Tryptophan</td>
<td>500</td>
<td>0.77</td>
<td>0.1 (−)</td>
</tr>
<tr>
<td>L-Kynurenine</td>
<td>100</td>
<td>0.70</td>
<td>7.8 (+)</td>
</tr>
<tr>
<td>Anthranilic acid</td>
<td>100</td>
<td>0.72</td>
<td>2.1 (+)</td>
</tr>
<tr>
<td>Catechol</td>
<td>100</td>
<td>0.74</td>
<td>0.1 (−)</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>500</td>
<td>0.80</td>
<td>0.1 (−)</td>
</tr>
<tr>
<td>Indole</td>
<td>500</td>
<td>0.76</td>
<td>0.1 (−)</td>
</tr>
<tr>
<td>D-Tryptophan</td>
<td>100</td>
<td>0.76</td>
<td>4.6 (+)</td>
</tr>
<tr>
<td>Kynurenic acid</td>
<td>500</td>
<td>0.74</td>
<td>0.1 (−)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (µM)</th>
<th>Growth (OD₆₅₀)</th>
<th>Hemolytic activity (H.U./ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DL-5-Fluorotryptophan</td>
<td>4</td>
<td>0.77</td>
<td>2.4 (+)</td>
</tr>
<tr>
<td>DL-6-Fluorotryptophan</td>
<td>4</td>
<td>0.63</td>
<td>1.8 (+)</td>
</tr>
<tr>
<td>3-Hydroxy-DL-kynurenine</td>
<td>100</td>
<td>0.77</td>
<td>0.1 (−)</td>
</tr>
<tr>
<td>2,3-Dihydroxybenzoic acid</td>
<td>500</td>
<td>0.74</td>
<td>0.1 (−)</td>
</tr>
</tbody>
</table>

* For inducers, concentrations cited are the lowest tested that still give a response; for noninducers, concentrations cited are the highest tested without growth inhibition.

a (−), Hemolytic; (+), nonhemolytic. H.U., Hemolytic units.