Research Note

Hydrophobicity, Cell Adherence, Cytotoxicity, and Enterotoxigenicity of Starved *Vibrio parahaemolyticus*

HIN-CHUNG WONG* and CHIA-NI CHANG

Department of Microbiology, Soochow University, Taipei, Taiwan 111, Republic of China

MS 04-161: Received 13 April 2004/Accepted 30 August 2004

ABSTRACT

*Vibrio parahaemolyticus* is a ubiquitous gram-negative enteropathogenic bacterium that may encounter starvation or other environmental stresses during food processing or human infection. Pathogenic *V. parahaemolyticus* ST550 cultures starved in modified Morita mineral salt solution with 3 or 0.5% NaCl exhibited similar resistance against challenges of environmental stresses. Changes in virulence of the starved *V. parahaemolyticus* was determined using HEp-2 cell culture and suckling mouse assay. The starved cells exhibited greater cell adherence and hydrophobicity than did the cells in exponential growth phase. Expression of virulence in terms of cytotoxicity and mouse lethality was lower in the starved cells than in the exponential-phase cells at the same postinfection time. An additional 1 h of in vitro or in vivo incubation was required to enable these starved cells to reach the same cytotoxicity and mouse lethality levels as exhibited by the exponential-phase cells.

*MATERIALS AND METHODS*

**Cultures and media.** *V. parahaemolyticus* ST550 is a serotype O4:K13 and KP clinical strain that was isolated in Thailand (17). It was maintained in tryptic soy broth (TSB; Difco, Becton Dickinson, Sparks, Md.) with 3% NaCl and 10% glycerol at −85°C and cultured in TSB with 3% NaCl or in tryptic soy agar (Difco, Becton Dickinson) with 3% NaCl and incubated at 25°C.

**Preparation of starvation-adapted cells.** An Erlenmeyer flask that contained 100 ml of TSB with 3% NaCl was inoculated with 10⁸ bacterial cells from a 16-h culture and incubated at 25°C in a static state for 4.5 h. A 100-ml volume of bacterial cells in the exponential growth phase was harvested by centrifugation at 6,000 × *g* for 15 min and washed twice in equal volumes of MMS with 0.5% NaCl (15). Bacterial cells were resuspended in MMS with 0.5% NaCl to yield a final concentration of 10⁷ CFU/ml. Freshly prepared cells were used as exponential-phase controls. The starved cells were prepared by suspending the bacterial cells in MMS medium with 0.5% NaCl at 25°C for 24 h. The bacterial density was determined by absorbance at 600 nm using a calibration curve determined by the plate count method.

**Determination of hydrophobicity.** The hydrophobicity of the exponential-phase or starved cells was determined using the xylene method (3).

**Determination of cell adherence and cytotoxicity.** HEp-2 cells (5 × 10⁵ cells per ml) were cultured to confluent phase on 12-mm-long glass slides located at the bottom of the wells of a 24-well plate in minimum essential medium with 10% fetal bovine serum at 37°C for 18 h in a 5% CO₂ incubator. The adherence of the bacterial cells to the HEp-2 cells was determined following a method described elsewhere (3). The cytotoxicity was determined following the procedures of Purven et al. (11). Bacteria (10⁷ CFU/ml) were added to each of the cell culture wells and incubated at 37°C for 4, 5, or 6 h and then washed with phosphate-buffered...
TABLE 1. Cell adherence and cytotoxicity of starved V. parahaemolyticus ST550 cells

<table>
<thead>
<tr>
<th></th>
<th>Cell adherence (no. of bacteria/HEp-2 cell)</th>
<th>% HEP-2 cells killed at:</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST550 cells</td>
<td></td>
<td>4 h</td>
</tr>
<tr>
<td>Exponential phase</td>
<td>10.7 ± 1.2</td>
<td>44.3 ± 2.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>59.3 ± 2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>72.7 ± 2.3</td>
</tr>
<tr>
<td>Starved</td>
<td>30.3 ± 3.8</td>
<td>29.6 ± 1.7b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>45.8 ± 2.1b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>63.3 ± 2.2b</td>
</tr>
</tbody>
</table>

a Data are means ± standard errors.

b Significantly different by t test at P < 0.05.

determined immediately before the death of the animals at

Exponential phase 4.8
MMS medium (control) 0 27
Starved, experiment 1 5.9 ± 0.3c
Exponential phase 4.8 ± 0.1
Starved, experiment 2 6.6 ± 0.4c

a Data are means ± standard errors.

b ND, not determined.

c Significantly different by t test at P < 0.05.

saline (PBS), fixed in 70% methanol, washed again in PBS, stained with 2% Giemsa solution for 20 min, washed again with PBS, dried in air, and observed under a microscope. That part of the microscopic field in which 50% or more of the monolayer was destroyed (estimated visually) was regarded as positive for cytotoxicity; 50 randomly selected fields on each glass plate were examined.

Animal assays. The mouse lethality and enterotoxigenicity of the exponential-phase or starved cells were determined by using suckling mice (17). A 0.1-ml aliquot of bacterial suspension in MMS with 0.5% NaCl (10⁷ bacterial cells per ml) with 15 µl of 1% Evan blue was injected into the stomach of each mouse. Each group consisted of six mice. The time of death of each mouse was recorded, and surviving mice were sacrificed at 2.5 h (exponential phase) or 3 h (starved) postinfection, and the intestine/whole body ratio of each mouse was determined. The intestine of each mouse was homogenized, and the population of V. parahaemolyticus was counted by serial dilution and the plate counting method on thiosulfate–citrate–bile salt–sucrose agar (Difco, Becton Dickinson) incubated at 37°C for 24 h.

Statistical analysis. The means of the exponential-phase and starved cell groups in each experiment were analyzed by performing a t test using the SPSS for Windows Network v. 11.0 (SPSS Inc., Chicago, Ill.).

RESULTS AND DISCUSSION

Environmental stresses regulate the expression of various bacterial phenotypes, including virulence factors (14). Starvation is the most extensively investigated phenomenon. Under starvation, gram-negative bacilli such as V. parahaemolyticus (8) usually undergo morphological changes from rod-shaped to small and spherical in a short time, accompanied by physiochemical changes such as changes in cell surface components (2) or in expression of extracellular enzymes (1) or intracellular protective enzymes (12). Loss of biological traits also has been observed in bacteria grown under starvation conditions for long periods (6). The key factor, RpoS, which is a starvation-induced alternative sigma factor in gram-negative bacteria, governs the expression of many virulence factors in enteric or other bacteria and is important in resisting stress (7). Nevertheless, a direct link between starvation and virulence has not been demonstrated conclusively (12).

In V. parahaemolyticus, acid adaptation increased the enteropathogenicity, as assayed using the suckling mouse model (17), and heat shock at 42°C promoted the production of thermostable direct hemolysin, the major virulence factor in this pathogen (18). The depletion of iron from the culture medium also promoted cell adherence and the production of thermostable direct hemolysin (3). The change in virulence of starved V. parahaemolyticus cells has not been investigated.

When V. parahaemolyticus was resuspended in MMS with 0.5% NaCl and incubated at 4°C, the bacteria entered into a viable but nonculturable state in about 35 days. When the bacterial cells were starved at 25°C prior to being treated at 4°C, the cells remained viable and culturable for several weeks (19). A special physiological state would have been reached during the starvation to maintain culturability and resistance to environmental stresses (15). The same starvation conditions were used in the present study. V. parahaemolyticus exhibited changes in its virulence, as determined by cytotoxicity, cell adherence, and enterotoxigenicity assays. HEP-2 adherence was markedly higher in the starved cells than in the exponential-phase cells (Table 1). The cytotoxicity of the starved cells was significantly lower than that of the exponential-phase cells at the same postinfection time point, but the cytotoxicity of both cell types increased with incubation time (Table 1). Duplicate animal assays were conducted for the starved cells, and both experiments produced similar results. The starved cells were less lethal than the exponential-phase cells at the same post-infection time point and required a longer incubation time to kill the infected animals (Table 2). The enterotoxigenicity determined immediately before the death of the animals at

TABLE 2. Mouse lethality, enterotoxigenicity, and hydrophobicity of starved V. parahaemolyticus ST550 cells

<table>
<thead>
<tr>
<th>Animal assay</th>
<th>Time of mouse death (h)a</th>
<th>Intestine/body wt ratio (×10⁻³)a</th>
<th>No. of bacteria in intestine homogenate (log CFU/g)</th>
<th>Hydrophobicityb</th>
</tr>
</thead>
<tbody>
<tr>
<td>ST550 cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMS medium (control)</td>
<td>0</td>
<td>27 ± 7</td>
<td>0</td>
<td>NDb</td>
</tr>
<tr>
<td>Exponential phase</td>
<td>4.8 ± 0.1</td>
<td>36 ± 6</td>
<td>5.72</td>
<td>0.56 ± 0.03</td>
</tr>
<tr>
<td>Starved, experiment 1</td>
<td>5.9 ± 0.3c</td>
<td>49 ± 7</td>
<td>4.91</td>
<td>0.88 ± 0.14c</td>
</tr>
<tr>
<td>Starved, experiment 2</td>
<td>6.6 ± 0.4c</td>
<td>41 ± 9</td>
<td>5.61</td>
<td>0.76 ± 0.04c</td>
</tr>
</tbody>
</table>

a Data are means ± standard errors.

b ND, not determined.

c Significantly different by t test at P < 0.05.
2.5 or 3 h postinfection in the exponential-phase or starved cells, respectively, revealed that the intestine/body weight ratios and the in vivo population of \textit{V. parahaemolyticus} in both groups were similar (Table 2). The enterotoxicogenicity results suggest that inherited virulence does not change during a short starvation period (1 day).

The conditions of growth also regulate the hydrophobicity of the bacterial surface, but hydrophobicity varies across species and even strains. \textit{Salmonella} Typhimurium cells starved for about 1 month exhibited reduced hydrophobicity (5). In this study, the hydrophobicity of the starved \textit{V. parahaemolyticus} markedly exceeded that of the exponential-phase cells (Table 2). The change in surface properties may be attributed to the increase in cell adherence of the starved cells.

Expression of virulence factors were affected by starvation in \textit{V. parahaemolyticus}. However, this effect probably is transient; about one additional hour of in vitro or in vivo incubation was required for starved cells to return to their normal growing state and reach the same cytotoxicity and mouse lethality levels as the exponential-phase cells.

**ACKNOWLEDGMENT**

This study was supported by a grant (NSC91-2313-B-031-004) from the National Science Council of the Republic of China.

**REFERENCES**


