Enterobacterial Autoinducer of Growth Enhances Shiga Toxin Production by Enterohemorrhagic Escherichia coli

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The addition of the enterobacterial autoinducer of growth to nutrient-poor minimal medium markedly accelerated the exponential growth rates of strains of enterohemorrhagic Escherichia coli but had little or no effect on maximal cell densities in stationary phase. Growth in the presence of the autoinducer resulted in an approximately twofold enhancement in Shiga toxin production.

Mammalian signaling molecules such as the catecholamine neuroendocrine hormone L-norepinephrine (NE) can stimulate the growth of a number of pathogenic bacteria and, in some cases, the expression of virulence properties (1, 2, 6, 7, 9, 10, 12, 17). In addition, transient exposure of Escherichia coli to NE in a serum-containing minimal medium induced the production of a heat-stable autoinducer of growth (AI), which elicited responses similar to those with NE, including induction of more AI (11). Similar effects were later observed for a range of enterobacterial species (4). In the case of E. coli, the mechanism of growth stimulation by NE includes facilitation of iron acquisition from the mammalian iron-binding proteins transferrin and lactoferrin (5). However, the activity of AI is not confined to bacterial growth under the iron-limited conditions of a serum-containing medium. We recently demonstrated re-suscitation by enterobacterial AI of highly stressed populations of pathogenic isolates of enterohemorrhagic E. coli (EHEC) and Salmonella enterica serovar Typhimurium in iron-containing nutrient-rich media (16). AI also enhanced the sensitivity and speed of enrichment of Bacillus cereus and Bacillus anthracis spores in both serum-supplemented medium and buffered peptone water (15). The present paper provides another example of iron-independent AI activity, with important practical implications for the detection of Shiga toxin-producing EHEC strains.

A new nutrient-poor minimal medium (NPMM) formulation that avoids the use of antibiotics and bile salts as selective supplements has been developed for enrichment of EHEC from clinical, food, and environmental samples (W. Voigt, A. Fruth, H.-H. Sonneborn, H. Tschäpe, and R. Reissbrodt, Abstr. VTEC 2003 Meet., p. 149, 2003). This medium comprises 100 mM K2HPO4 (pH 7.2 ± 0.1) containing trace elements (40 mg/liter of MgSO4 · 7H2O, 4 mg/liter of ferric ammonium citrate, 6 mg/liter of MnSO4 · 4H2O, vitamins [0.2 mg/liter each] of pyridoxal-HCl, thiamine, and nicotinic acid amide), and pancreatic casein peptone (0.2% [wt/vol]) as a source of amino acids (14). During developmental work, it was observed that aspartate, glutamate, and serine were significantly depleted during the growth of EHEC strains, as measured by capillary electrophoresis of samples taken before and after growth. Therefore, the medium was additionally supplemented with L-aspartic acid (0.08% [wt/vol]), L-glutamic acid (0.12% [wt/vol]), and L-serine at various concentrations, as described below. The medium was supplemented as required with 50 µM NE (Sigma Chemical Co.) or with 2% (vol/vol) AI prepared as previously described (4, 8, 16). Briefly, serum-SAPI medium (2.77 mM glucose, 6.25 mM NH4NO3, 1.84 mM KH2PO4, 3.35 mM KCl, 1.01 mM MgSO4, and 30% [vol/vol] adult bovine serum, pH 7.5) containing 50 µM NE was inoculated at approximately 100 to 1,000 CFU/ml with the producing strain and incubated overnight at 37°C in a humidified 5% CO2 atmosphere. Bacteria were pelleted by centrifugation (6,000 × g for 15 min), and the culture supernatants were filter sterilized and stored at −20°C until required. Sterile preparations were serially diluted in fresh sterile SAPI medium, and samples of each dilution were added at 2% (vol/vol) to serum-SAPI medium inoculated with 100 to 1,000 CFU/ml of an indicator strain. The dilution that promoted bacterial growth after overnight incubation at 37°C to an optical density at 620 nm (OD620) of 0.4, which represents 10⁸ CFU/ml, was used as a supplement to NPMM, as described below. For the experiments reported here, we prepared AI from cultures of a strain that we had previously reported to be Yersinia ruckeri (16) but subsequently confirmed (on the basis of the Bactid-System [CDC, Atlanta, Ga.]) to be E. coli. It should be noted, however, that virtually any strain of E. coli or any other enterobacterial species that produces enterobactin can be used both for production of AI and as an indicator strain for AI activity.

The EHEC strains analyzed in this study were from the culture collection of the Robert Koch Institute, Wernigerode, Germany. Bacteria from overnight nutrient agar cultures were inoculated into NPMM at approximately 2 × 10⁵ CFU/ml and incubated at 37°C with horizontal shaking at 150 rpm. Culture growth (OD620) was monitored in a Bioscreen C apparatus (Thermolabsystems, Helsinki, Finland) (16), and Shiga toxin production was determined with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (r-Biopharm, Darmstadt, Germany) and reported as the OD₄₉₀. Two-tailed Student’s t test was used to determine the differences in toxin production between culture conditions; differences were considered statistically significant when P values were <0.05.

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Figure 1 illustrates a Bioscreen C analysis of the growth of *E. coli* strain 97-10085 in the presence or absence of AI. Supplementation with AI significantly accelerated exponential growth, such that between 7 and 10 h, the doubling time of the culture in the presence of AI was consistently approximately half that in the absence of AI. After prolonged incubation, however, OD620 levels in AI-free medium and AI-supplemented medium were similar. This is in contrast with the situation in serum-SAPI medium (11), in which growth in the absence of AI is limited to between 100- and 1,000-fold, primarily (but perhaps not exclusively) because iron availability is restricted by the presence of serum transferrin. Indeed, AI was so designated because of its ability to induce bacterial growth under the specific conditions of serum-SAPI medium (11). Under the relatively iron-rich conditions used in this study, however, the activity of AI is seen as an enhancement of the growth rate rather than the final cell density.

Table 1 shows the effects of supplementation of NPMM with NE or AI on Shiga toxin production by EHEC strains and comparison with a commercially available EHEC diagnostic medium.

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Serovar</th>
<th>Shiga toxin(s)</th>
<th>Shiga toxin production (OD450)* in:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>NPMM</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>No supplement +NE +AI</td>
</tr>
<tr>
<td>97-04951</td>
<td>O8:H1</td>
<td>Stx1, Stx2</td>
<td>3.9</td>
</tr>
<tr>
<td>01-03554</td>
<td>O8:K1:H1</td>
<td>Stx2</td>
<td>0.4</td>
</tr>
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<td>O157:H7</td>
<td>Stx2</td>
<td>0.2</td>
</tr>
<tr>
<td>01-02906-2</td>
<td>O157:H7</td>
<td>Stx1</td>
<td>0.3</td>
</tr>
<tr>
<td>97-10085</td>
<td>O157:H7</td>
<td>Stx1, Stx2</td>
<td>1.9</td>
</tr>
</tbody>
</table>

* Determined by a commercial ELISA kit. Asterisks indicate significance, with *P* values of <0.05.

The results shown are averages of data from at least three independent experiments.

![FIG. 2. Growth and Shiga toxin production of *E. coli* strain 97-10085 in NPMM containing increasing amounts of l-serine, as indicated (white bars), and additionally supplemented with 2% AI (black bars) after 24 h of incubation at 37°C. (a) Culture growth monitored by OD620 in a Thermolab systems Bioscreen C apparatus. (b) Shiga toxin production monitored by OD450 with a commercially available ELISA kit. The results shown are averages of data from at least three independent experiments.](image-url)
of AI resulted in a slight but not statistically significant reduc-
tion ($P > 0.05$) in the maximum OD$_{620}$ level achieved after 24 h of incubation at each concentration of L-serine (Fig. 2a).

Analysis of toxin production in the same cultures indicated that adding increasing concentrations of L-serine resulted in only small increases in Shiga toxin production but that supplementation with 2% (vol/vol) AI markedly enhanced Shiga toxin production at all L-serine concentrations tested (Fig. 2b). Similar data were obtained with the nine other strains listed in Table 1 (data not shown).

It has been proposed that AI preparations contain entero-
bactin and its breakdown products, the so-called enterobactin complex formed by enterobactin-producing strains under conditions of iron limitation (3, 13, 15). In the iron-restricted environment of serum-SAPI medium, the presence of sid-
erophores would clearly explain enhanced growth and concomi-
tant toxin expression, as reported by Lyte and coworkers (8, 11). It is unlikely, however, that the iron supply alone is re-
sponsible for the properties of AI reported here, first because the medium used is sufficiently iron-rich that an additional iron source is not required for growth, and second because the toxin Stx2, which is produced by many of the EHEC strains tested here, is not iron regulated (18). High-performance liquid chromato-
graphy analysis of the AI preparations used in this study indicated that they do indeed contain components of the en-
terobactin complex (data not shown), but additional compo-
unds are also present, and further detailed analysis is ongo-
ing to determine their nature and activity.

In terms of the application of our work, Table 1 illustrates the effectiveness of NPMM supplemented with AI compared with a commercially available culture medium for EHEC di-
agnosis. Partially purified AI is very simple and cheap to pre-
pare. We therefore propose that AI-supplemented NPMM be
used routinely to enhance the sensitivity of Shiga toxin detec-
tion in food and environmental samples. This is especially relevant when speed of detection is an important consid-
eration. Any enhancement in toxin production will shorten the time required for unequivocal detection, which may be partic-
ularly valuable if a diagnostic result can be obtained in a single working day rather than after an overnight enrichment step.

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