Further Purification and Characterization of Heat-Stable Enterotoxin Produced by Yersinia enterocolitica

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Heat-stable enterotoxin (ST) of Yersinia enterocolitica was produced under defined conditions. It was first detected in the culture supernatant of the late-logarithmic phase of growth and increased linearly during the stationary phase of growth. The ST level became maximum at the decline phase of growth, and the ST was not detected in the lysate of bacteria obtained from the decline phase of growth. The ST was extensively purified from the culture supernatant, and about a 1,905-fold purification was achieved with a yield of 8.9%. The minimal effective dose of the purified ST was approximately 25 ng in the suckling mouse assay. The purified ST gave a single 280-nm absorbing peak on polyacrylamide disc gel electrophoresis and had a maximum absorption at 272 nm, and its molecular weight was 9,700 by Sephadex G-75 superfine gel filtration. The biological activity of the purified ST was lost by treatment with 2-mercaptoethanol, suggesting that the ST contained disulfide bridges in the molecule which were required for the development of toxic activity. The purified ST was heat stable at 100°C for 10 min between pH 2.2 and 8.0, but not at pH values greater than 9.0 or in 2 N HCl. The treatment of the ST with trypsin resulted in a retarded elution of the ST activity by Sephadex G-75 superfine gel filtration and a passage through a UM-20 membrane filter.

Yersinia enterocolitica is an important cause of bacterial gastroenteritis in children (5, 11). One potential pathogenic property of the organism as an enteric pathogen is heat-stable enterotoxin (ST) production. Preliminary investigations indicated that Y. enterocolitica ST was similar to Escherichia coli ST with respect to suckling mouse activity, accumulation of cyclic guanosine 3′,5′-monophosphate in the intestine, and failure to induce morphological changes in Chinese hamster ovary cells (2, 13, 15–17). Recently, we reported that the STs of Y. enterocolitica and E. coli are immunologically cross-reactive, although the heat stability and molecular weight of the partially purified Y. enterocolitica ST are different from those of the E. coli ST (14). In this paper, we describe the method for further purification of Y. enterocolitica ST and demonstrate its physicochemical characteristics for elucidating the active center of Y. enterocolitica ST.

MATERIALS AND METHODS

Bacterial strains and cultivation. Y. enterocolitica 23 and CY medium (Casamino Acids and yeast extract broth supplemented with glucose, pH 8.5) were used for the production of ST as described previously (14), except that the bacteria were cultured in a 5-liter minijar fermentor (Mituwa Rigagaku Kogyo Co., Ltd., Osaka, Japan), equipped with a pH-control unit. Preculture was prepared by inoculating the frozen culture of Y. enterocolitica 23 into 50 ml of CY medium in a 500-ml rotary bottle and by incubating at 25°C for 24 h with constant rotation (200 rpm). Then, the preculture was seeded into the jar fermentor containing 3 liters of fresh CY medium, and the organisms were grown at 25°C for 48 h with constant aeration (3 liters per min) and stirring (400 rpm). The pH of the medium was controlled by 1 N HCl so as not to rise above 8.5. Samples were drawn at intervals to monitor the growth of bacteria and the production of ST.

Assay for ST. ST activity was assayed in suckling mice as reported previously (13). Briefly, 0.1-ml samples were administered by gastric tube into the stomachs of 2- to 3-day-old suckling mice (Icr, Nippon Clea Co., Japan), with 0.001% Evans blue dye as a marker. The mice were kept for 1 h at 25°C and were then sacrificed by inhalation of chloroform, after which the entire intestines were removed. A ratio of intestine weight to body weight of 0.083 was considered to be a positive response, and 1 U was defined as the minimum amount of protein required to give a positive response. Five mice were used for the determination of enterotoxin titer for each sample.

Purification of Y. enterocolitica ST. Partial purification of Y. enterocolitica ST was achieved as described previously (14). The partially purified ST obtained from 12-liter culture filtrate was divided into two equal parts. Each part (8.95 mg of protein) dissolved in 10 mM Tris-hydrochloride buffer (pH 7.2) was chromatographed on a column (2 by 40 cm) of DEAE-Sephalac...
(Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) equilibrated with the same buffer. The adsorbent was washed with 400 ml of 10 mM Tris-hydrochloride buffer (pH 7.2) containing 100 mM NaCl. Then elution was performed with 800 ml of a linear gradient of 100 to 300 mM NaCl in the same buffer. Fractions containing ST activity were collected, concentrated by using a rotary evaporator, and dialyzed against 10 mM Tris-hydrochloride buffer (pH 7.2). The material (5.3 mg of protein) was applied to a Bio-Gel P-10 (Bio-Rad Laboratories, Richmond, Calif.) column (2 by 100 cm), equilibrated with 10 mM Tris-hydrochloride buffer (pH 7.2), and eluted with the same buffer.

Polyacrylamide disc gel electrophoresis. Polyacrylamide disc gel electrophoresis was carried out in 7 or 14% acrylamide gel (pH 9.5 as the running pH) by the method described by Davis (4). Bromophenol blue dye was used as a marker. After electrophoresis of the purified ST, some of the gels were stained with Coomassie brilliant blue R, and others were scanned at 280 nm with a chromatogram scanner (Shimadzu CS-90, Shimazu Corp., Kyoto, Japan). The scanned gels were then cut into 3-mm-thick sections, and each section was extracted with 0.5 ml of 10 mM Tris-hydrochloride buffer (pH 7.2) for about 40 h. The ST activity in the extract was examined as described above.

Molecular weight determination. The molecular weight of the purified ST was determined by Sephadex G-75 superfine (Pharmacia) gel filtration. The column (2 by 117 cm) was eluted with 10 mM Tris-hydrochloride buffer (pH 7.2). Cytochrome c (molecular weight, 12,500), chymotrypsinogen A (molecular weight, 25,000), hen egg albumin (molecular weight, 45,000), and bovine serum albumin (molecular weight, 68,000) served as markers for the estimation of molecular weight.

Test for pH stability. A sample of the purified ST (40 U) was heated at 100°C for 10 min in 1 ml each of various concentrations of hydrochloric acid (0.5, 1, 2, and 3 N), 50 mM glycine-HCl buffers (pH 2.2 and 3.0), 50 mM citrate buffers (pH 4.0 and 5.0), 50 mM phosphate buffers (pH 6.0, 7.0, and 8.0), 50 mM glycine-NaOH buffers (pH 9.0 and 10.0), and 50 mM carbonate-bicarbonate buffer (pH 10.7). After being heated, each toxin solution was adjusted to pH 7.0 with NaOH or HCl and dialyzed against distilled water by using Spectra/por 6 membrane tubing (Spectrum Medical Industries, Inc., Los Angeles, Calif.) which filters out molecules with molecular weights of less than 1,000. Finally, the volume was adjusted with distilled water to 2 ml.

Treatment of purified ST by 2-mercaptoethanol. The purified ST (5 µg) was incubated at either 37°C or 100°C for 10 min in 1 ml of 10 mM Tris-hydrochloride buffer (pH 7.2) containing 200 µmol of 2-mercaptoethanol and then dialyzed against distilled water with or without alkylation. Alkylation was performed by adding 200 µmol of monooiodoacetic acid and by incubating at 37°C for 60 min in the dark (8).

Trypsinization of purified ST. The purified ST (500 U) was incubated with 125 µg of trypsin (Mochida Pharmaceuticals, Tokyo, Japan) at an ST-to-enzyme ratio of 1:10 (wt/wt). After an incubation of 60 min at 37°C (pH 7.2), the reaction was stopped by boiling the mixture for 10 min. The reaction mixture was applied to a Sephadex G-75 superfine column (2 by 117 cm) equilibrated with 10 mM Tris-hydrochloride buffer (pH 7.2), and the ST activity was eluted with the same buffer.

Samples of 50 ml of the trypsinized ST (60 U/ml) were concentrated to 25 ml on YM-5, UM-10, or UM-20 membrane filters (Amicon Corp., Lexington, Mass.). These membranes filtered out molecules with a molecular weights of 5,000, 10,000, or 20,000, respectively. Enterotoxigenic activity of each filtrate was assayed by using suckling mice.

Protein, carbohydrate, and endotoxin determination. Protein was determined by the method of Lowry et al. (12) with bovine serum albumin as a standard. Total carbohydrate was determined by the phenol-sulfate technique with glucose as a standard (10), and endotoxin activity was estimated by limulus assay (21).

RESULTS

Relationship between toxin production and growth of Y. enterocolitica. The elaboration of ST into culture medium and the growth rate of Y. enterocolitica at 25°C are shown in Fig. 1. Under the conditions used here, the ST activity was not detected in the culture supernatant obtained from the logarithmically growing culture. It was measurable at the late-logarithmic phase and reached maximum at the decline phase of growth. The ST activity was detected only in the culture supernatant but not in the extract of the 48-h grown cell.

Purification of Y. enterocolitica ST. The partially purified ST was rechromatographed on a DEAE-Sephacel column. The ST activity was eluted at an approximately 200 mM NaCl, and the fractions containing ST activity were collected, concentrated by evaporation, and dialyzed against 10 mM Tris-hydrochloride buffer (pH 7.2). This material was subjected to gel filtration on Bio-Gel P-10 (Fig. 2). The ST activity was associated with the first eluted peak. This final

FIG. 1. Time course of the ST production. The ST activity of the culture supernatant was determined by the suckling mouse assay as described in the text. Cell growth is shown by the turbidity of culture. Symbols: ●, units of ST activity; ▲, absorbance of whole culture at 600 nm.
material was colorless and used as the purified ST in the further studies.

**Recovery of ST activity during purification.**

The recovery of toxin at each stage of the purification from 12-liter of culture filtrate is shown in Table 1. The minimal effective dose of the purified ST was about 25 ng, and the purified ST did not contain carbohydrate and endotoxin. The specific activity of the ST increased about 1,905-fold, and the final recovery was about 8.9%.

**Physicochemical properties of the purified ST.**

When the purified ST (30 µg of protein) was analyzed on polyacrylamide disc gel electrophoresis, the activity of ST was recovered from the gel near the position of marker dye, whereas no protein bands were visible after staining of the gel with Coomassie brilliant blue R. Therefore, the gel was scanned at 280 nm immediately after electrophoresis of the purified ST. A single 280-nm absorbing peak was observed with an Rf of approximately 1.0 in a 7% gel and 0.9 in a 14% gel (Fig. 3). The activity of ST recovered from the sliced gels was superimposable with the 280-nm absorbing peak.

The absorption spectrum of the purified ST has a maximum at about 272 nm and a minimum at 250 nm. The molecular weight of the purified ST was approximately 9,700 by gel filtration (Fig. 4), but the purified ST was unable to pass through the UM-20 membrane which filters out molecules with molecular weight of 20,000 (Table 4). As we reported previously on the partially purified ST (14), the purified ST dissolved in distilled water was heat stable at 100°C and at 121°C for 20 min. However, the purified ST was inactivated by boiling for 10 min in either alkaline medium (pH 9.0) or extremely acidic medium (2 N HCl) (Fig. 5 and Table 2).

Purified ST (200 U; equivalent to 5 µg of protein) was reduced in the presence of 200 µmol of 2-mercaptoethanol at either 37 or 100°C for 10 min, and its activity was measured without dialysis. Reduction of the purified ST resulted in a complete loss of the activity at 100°C but only partial loss at 37°C (Table 3). When the reduced ST was dialyzed without alkylation 5% of the original activity was recovered, whereas no ST activity was detected after alkylation.

We reported previously that trypsin digestion did not affect the activity of the partially purified ST (14). Similarly, the activity of the purified ST was not destroyed by treatment with trypsin. But the analysis of the trypsinized ST by gel

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**TABLE 1. Recovery of ST activity during purification**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein (mg)*</th>
<th>Total activity (U)*</th>
<th>Specific activity (U/mg)</th>
<th>Relative activity</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture supernatant</td>
<td>58,641.00</td>
<td>1,231,460</td>
<td>21</td>
<td>1.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Protamine sulfate- treated ST</td>
<td>1,873.70</td>
<td>904,796</td>
<td>483</td>
<td>23.0</td>
<td>73.5</td>
</tr>
<tr>
<td>DEAE-Sepharose column eluate</td>
<td>379.20</td>
<td>597,258</td>
<td>1,575</td>
<td>75.0</td>
<td>48.5</td>
</tr>
<tr>
<td>Hydroxylapatite column eluate</td>
<td>58.20</td>
<td>270,921</td>
<td>4,655</td>
<td>221.7</td>
<td>22.0</td>
</tr>
<tr>
<td>Sepherose S-200 superfine column eluate</td>
<td>17.90</td>
<td>153,933</td>
<td>8,600</td>
<td>409.5</td>
<td>12.5</td>
</tr>
<tr>
<td>DEAE-Sepharose column eluate</td>
<td>5.30</td>
<td>136,692</td>
<td>25,791</td>
<td>1,228.1</td>
<td>11.1</td>
</tr>
<tr>
<td>Bio-Gel P-10 column eluate</td>
<td>2.74</td>
<td>109,600</td>
<td>40,000</td>
<td>1,904.8</td>
<td>8.9</td>
</tr>
</tbody>
</table>

* Protein content was measured by the method of Lowry et al. (12).

* One unit is defined as the minimal amount giving an intestine weight-to-body weight ratio of 0.083 at 3 h after administration of the sample.
filtration and by ultrafiltration suggested that a small active fragment carrying a toxic activity was separated by treatment with trypsin. The ST activity of trypsinized sample was eluted at an included volume of the Sephadex G-75 superfine column, but the activity of untreated ST was eluted between the void and the included volume of the gel (Fig. 6). The result of ultrafiltration is shown in Table 4. The untreated purified ST did not pass through these membrane filters whereas the ST treated with trypsin passed through the UM-20 membrane filter but not the YM-5 or UM-10 membrane filters.

DISCUSSION

Recently, we partially purified the Y. enterocolitica ST from the supernatant of the 48-h culture and demonstrated that Y. enterocolitica ST immunologically cross-reacted with E. coli ST (14). However, the conditions that are suitable for the production of Y. enterocolitica ST

![Graph](image)

FIG. 3. Polyacrylamide disc gel electrophoresis of the purified Y. enterocolitica ST. Polyacrylamide disc gel electrophoresis was carried out as described in the text. (A) 7% gel, running pH, 9.5; (B) 14% gel, running pH, 9.5. After electrophoresis, the gel was scanned at 280 nm (— — ) and then was cut into 3-mm-thick sections, which were extracted with 0.5 ml of 10 mM Tris-hydrochloride buffer (pH 7.2) for about 40 h. Then, 0.1 ml of the extract was administered to the suckling mouse, and the intestine-to-body weight (IW/BW) ratio was determined after 3 h (●).

![Graph](image)

FIG. 4. Molecular weight determination of the purified Y. enterocolitica ST by Sephadex G-75 superfine gel filtration.

![Graph](image)

FIG. 5. Effect of pH on activity of the purified Y. enterocolitica ST. The purified Y. enterocolitica ST (40 U) was dissolved in 1.0 ml of 50 mM concentrations of various kinds of buffer. After heating at 100°C for 10 min, the pH of the sample was adjusted to 7.0, and the sample was dialyzed against distilled water. Then, 0.1 ml of the sample was administered to the suckling mouse, and the intestine-to-body weight (IW/BW) ratio was determined as described in the text. Values are means ± standard error of five determinations.

![Graph](image)

TABLE 2. Effect of HCl treatment on activity of purified Y. enterocolitica ST

<table>
<thead>
<tr>
<th>Conc of HCl (N)</th>
<th>Intestine wt/body wt ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.093 ± 0.003</td>
</tr>
<tr>
<td>0.5</td>
<td>0.090 ± 0.005</td>
</tr>
<tr>
<td>1.0</td>
<td>0.085 ± 0.003</td>
</tr>
<tr>
<td>2.0</td>
<td>0.060 ± 0.003</td>
</tr>
<tr>
<td>3.0</td>
<td>0.055 ± 0.004</td>
</tr>
</tbody>
</table>

* The purified ST (40 U) was heated at 100°C for 10 min in 1 ml each of various concentrations of HCl (0.5, 1.0, 2.0, and 3.0 N). After heating, each solution was adjusted to pH 7.0 with NaOH and dialyzed against distilled water. The volume of the sample was adjusted with distilled water to 2 ml, and the ST activity of the sample was assayed in suckling mice.

b Ratio of intestine to body weight of suckling mice at 3 h after administration of the sample. Values represent the mean ± standard error of five determinations.
are not yet fully understood. Johnson et al. (9) found that E. coli ST could be detected in the early-logarithmic phase of growth, and that pH control (pH 8.5) resulted in greater levels of ST at all stages of growth in Evans medium supplemented with 0.5% glucose. In this study, we demonstrated that Y. enterocolitica ST was not detected in the culture supernatant until the late-logarithmic phase of the pH-controlled culture. This may suggest that the control mechanism for the synthesis of E. coli ST and Y. enterocolitica ST is not the same. The accumulation of Y. enterocolitica ST in the culture supernatant continued to increase from the late-logarithmic phase to the decline phase of growth (40 h). The level of the Y. enterocolitica ST became maximum at 40 h and remained constant thereafter. We could not detect the ST activity in the extract of the 48-h cultured cell. Staples et al. (20) reported that agitation of the culture medium during growth was required for the effective production of E. coli ST. They also reported that the time of incubation and the composition of medium were also critical points in obtaining the purified E. coli ST. In the present study, pH control and constant aeration invariably resulted in a two- to threefold increase of the ST production compared with the supernatant obtained from the conventional culture. Further selection of the culture condition for Y. enterocolitica probably makes it easy to purify toxin.

Purification of the ST was accomplished by the method reported previously (14) with the two additional chromatographic separations. The purified Y. enterocolitica ST was a single peak on gel filtration and did not contain sugar and lipopolysaccharide. After polyacrylamide disc gel electrophoresis, purified Y. enterocolitica ST was eluted with a single 280-nm absorb-

<table>
<thead>
<tr>
<th>Conc of 2-mercaptoethanol (µmol/ml)</th>
<th>Temp during reduction (°C)</th>
<th>ST activity (U/ml)</th>
<th>None dialyzed</th>
<th>Dialyzed</th>
<th>Alkylated and dialyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>NT</td>
</tr>
<tr>
<td>200</td>
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<td>120</td>
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</tr>
<tr>
<td>200</td>
<td>100</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

* The purified Y. enterocolitica ST (200 U) was dissolved in 1.0 ml of 10 mM Tris-hydrochloride buffer (pH 7.2) containing 200 µmol of 2-mercaptoethanol per ml and heated at 37 or 100°C for 10 min. A part of the sample was dialyzed against distilled water by use of Spectra/por 6 membrane, and the other part was dialyzed similarly after alkylation with monoiodoacetic acid. The ST activity was assayed before and after dialysis in suckling mice.

b One unit is defined as the minimal amount giving an intestine-to-body weight ratio of 0.083 at 3 h after administration of the sample.

c NT, Not tested.

### FIG. 6. Gel filtration patterns of the purified Y. enterocolitica ST treated with trypsin and the untreated ST on Sephadex G-75 superfine column. The purified ST (500 U) treated with trypsin (●) and the untreated (□) were chromatographed separately on Sephadex G-75 superfine column (2 by 117 cm) as described in the text. Eluate was collected in 3.8-ml fractions. The eluate was administered to suckling mouse and the intestine-to-body weight (IW/BW) ratio was determined as described in the text. The void (V₀) and included volume (V₁) of the column were determined with blue dextran and NaCl, respectively.

### TABLE 3. Effect of 2-mercaptoethanol on activity of purified Y. enterocolitica ST

<table>
<thead>
<tr>
<th>Conc of 2-mercaptoethanol (µmol/ml)</th>
<th>Temp during reduction (°C)</th>
<th>ST activity (U/ml)</th>
<th>None dialyzed</th>
<th>Dialyzed</th>
<th>Alkylated and dialyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>NT</td>
</tr>
<tr>
<td>200</td>
<td>37</td>
<td>110</td>
<td>120</td>
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<td>200</td>
<td>100</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

### TABLE 4. Enterotoxigenic activity after ultrafiltration of the purified ST treated with trypsin

<table>
<thead>
<tr>
<th>Prepn</th>
<th>Membrane</th>
<th>Intestine wt/body wt ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated ST</td>
<td>YM-5</td>
<td>0.051 ± 0.003</td>
</tr>
<tr>
<td>UM-10</td>
<td>0.054 ± 0.006</td>
<td></td>
</tr>
<tr>
<td>UM-20</td>
<td>0.052 ± 0.005</td>
<td></td>
</tr>
<tr>
<td>ST treated with trypsin</td>
<td>YM-5</td>
<td>0.054 ± 0.004</td>
</tr>
<tr>
<td>UM-10</td>
<td>0.056 ± 0.006</td>
<td></td>
</tr>
<tr>
<td>UM-20</td>
<td>0.090 ± 0.007</td>
<td></td>
</tr>
</tbody>
</table>

* The purified ST treated with trypsin was subjected to ultrafiltration, and a resulting ultrafiltrate was administered to suckling mice.

b Ratio of intestine to body weight of suckling mouse at 3 h after administration of the sample. Values represent the mean ± standard error of five determinations.
ing peak and was not stained with Coomassie brilliant blue. These data show that the purified ST is almost homogenous. The minimal effective dose of the purified ST (25 ng by Lowry protein) in suckling mouse assay was high compared with that of the purified E. coli ST (1, 20, 22). The molecular weight of Y. enterocolitica ST was about 9,700 by Sephadex G-75 superfine gel filtration, whereas that of human E. coli ST was 1,972 by analysis of amino acid composition (20). The minimal effective dose of the latter was either 7.9 ng (by Lowry protein) or 2.9 ng (by dry weight) (20). However, trypsin split off a small toxic fragment from the purified Y. enterocolitica ST, and a resulting fragment was separated by gel filtration and neutralized by a specific antiserum against the purified Y. enterocolitica ST (data not shown). These data may account for the reason why the minimal effective dose of Y. enterocolitica ST is different from that of E. coli ST. The molecular weight of the purified Y. enterocolitica ST determined by gel filtration is to some extent contradictory to that expected from the ultrafiltration, but it is generally agreed that whether a molecule passes through a given ultrafiltration membrane is dependent not only on the molecular weight but also the spatial structure and the charge of molecule.

The purified Y. enterocolitica ST was stable at acidic pH values but not at pH values above 9 as E. coli ST (20, 22). Reduction by mercaptoethanol caused a loss of the activity dependent on its reaction temperature, and the activity was recovered partially by dialysis. No activity, however, was recovered after an alklylation. This suggests the presence of disulfide bridges, which are required for the development of biological activity. The presence of disulfide bridges would account for the heat and pH stability as reported for E. coli ST (20).

Zink et al. (23) found that the presence of a 41 × 10^6 plasmid absolutely correlated with tissue invasiveness (Positive Sérény test) in strains of Y. enterocolitica involved in an outbreak of human enteric disease, but that the ability for producing ST was not associated with any particular plasmid. They suggested that Y. enterocolitica ST production was encoded by chromosomal genes. Furthermore, Gemski et al. (6, 7) reported that a plasmid was associated with pathogenicity and V and W antigens but was not associated with the production of Y. enterocolitica ST. The production of E. coli ST, however, is controlled by plasmid DNA (18, 19), and Y. enterocolitica ST and E. coli ST are known to exchange plasmid DNA (3). Therefore, it is not clear whether Y. enterocolitica ST genes are always located on chromosomal genes. We are seeking the transmissible plasmid for Y. enterocolitica ST, which would answer the evolutionary question of E. coli ST and Y. enterocolitica ST and clarify the role of plasmid in pathogenesis.

LITERATURE CITED

