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ALEXANDER SULAKVELIDZE,1* ARNOLD KREGER,1 AARON JOSEPH,1 ROY M. ROBINS-BROWNE,2,3 ALESSIO FASANO,4 GEORGES WAUTERS,5 NORMA HARNETT,6 LOUIS DE'TOLLA,7,8 AND J. GLENN MORRIS, JR.1

Divisions of Hospital Epidemiology1 and Infectious Diseases,7 Department of Medicine, Division of Gastroenterology and Nutrition, Department of Pediatrics,4 and Department of Pathology,8 University of Maryland School of Medicine, Baltimore, Maryland 21201; Department of Microbiology and Immunology, Royal Children’s Hospital,2 and Department of Microbiology, University of Melbourne,3 Parkville, Victoria 3052, Australia; Unité de Microbiologie, Faculté de Médecine, Université Catholique de Louvain, Brussels, Belgium; and National Centre for Yersinia, Ontario Ministry of Health, Toronto, Ontario, Canada M5W 1R6

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*Yersinia bercovieri*, a recently identified *Y. enterocolitica*-like species, produces a heat-stable enterotoxin (designated YbST) which has biologic activity in infant mice and increases short circuit current in Ussing chambers. Although YbST has some properties in common with the heat-stable enterotoxins of *Y. enterocolitica* (YST I and YST II), it appears to be a novel toxin because (i) it was not neutralized by anti-YST I antiserum, (ii) YbST-neutralizing antiserum did not neutralize YST I, and (iii) *Y. bercovieri* strains did not hybridize with genetic probes for *yst I, yst II*, and other known enterotoxins.

*Yersinia enterocolitica* is a widely recognized enteric pathogen which causes a variety of gastrointestinal and systemic syndromes (2, 4, 5). Eight biogroups of *Y. enterocolitica* recently have been classified as separate species, at least one of which (*Y. kristensenii*) has been implicated as a possible cause of human disease (16). *Y. bercovieri* is one of the eight “new” *Y. enterocolitica*-like species and was previously designated *Y. enterocolitica* biogroup 3B. Strains of this species were first isolated by Bercovier et al. in 1978 (1), although the species was not named until 1988, when Wauters et al. (20) proposed the name *Y. bercovieri*. Since then, *Y. bercovieri* strains have been isolated primarily from patients with diarrhea, with additional isolates coming from raw foods and environmental samples (8, 20; and our data). In addition to the biochemical differences which accounted for their initial designation as *Y. enterocolitica* biogroup 3B, *Y. bercovieri* strains have chemically distinct O antigens (10) and have been found to be phylogenetically different from *Y. enterocolitica* when investigated by multilocus enzyme electrophoresis (8), pulsed-field gel electrophoresis (18), and M13 phage typing (19).

During our recent screening of *Y. enterocolitica* and *Y. enterocolitica*-like species for virulence markers, we found that boiled culture supernatant fluids (CSF) of two *Y. bercovieri* isolates (*Y. bercovieri* 6517 and 6519) obtained from the Republic of Georgia had striking enterotoxic activity in infant mice (17). Ninety-one additional isolates subsequently were obtained, and their CSF were tested for enterotoxic activity in infant mice by a standard procedure (6). Briefly, bacteria were grown with vigorous shaking at 28°C for 48 h in Trypticase soy broth (Difco Laboratories, Detroit, Mich.) supplemented with 0.6% yeast extract (TSB/YE), and 3- to 4-day-old CD-1 mice (four mice/group) were inoculated orogastrically with aliquots (0.1 ml) of CSF obtained by sequential centrifugation, membrane filtration (0.2-μm pore size), and heating (100°C; 10 min). The mice were kept at 28°C for no more than 4 h before euthanasia, and ratios of intestinal weight to remaining body weight were calculated for each mouse. Rations of <0.060 were considered negative, those in the range of 0.060 to 0.080 were considered borderline positive, and those of >0.080 were considered positive. CSF of YST I-producing *Y. enterocolitica* strains A2635 and 8081 (positive controls) elicited a mean ratio of 0.084, and TSB/YE and CSF of YST I-negative *Y. enterocolitica* 1114 gave a ratio of 0.047. All strains were tested at least twice. As shown in Table 1, heated CSF obtained from 69 strains of *Y. bercovieri* gave strongly positive responses, 14 strains were negative, and 10 isolates produced borderline positive results. In all cases the positive results were easily interpreted visually; a typical (not maximal) positive response is shown in Fig. 1.

CSF from 64 of the 69 strongly positive strains (93%) produced massive fluid accumulation and killed the mice within 2.5 to 3 h of inoculation. To our knowledge, death has not been observed during testing of CSF of YST I- and YST II-producing strains of *Y. enterocolitica* (12, 13, 15; our data). There was no correlation between enterotoxin-production and O serogroups of *Y. bercovieri*. On the other hand, there appeared to be a correlation between enterotoxigenic and lethal activities; i.e., all of the lethal CSF were enterotoxin-positive, and none of the enterotoxin-negative CSF were lethal. In addition, CSF obtained from the borderline positive and negative groups were not lethal. However, rigorous proof that the lethal activity of the CSF is a function of YbST must await characterization of the lethal activity of highly purified YbST preparations.

Light microscopy of the small intestines of infant mice treated with active CSF of *Y. bercovieri* revealed severe blunting of villi and moderate villus atrophy, exfoliation of enterocytes into the lumen, and an edematous lamina propria and
TABLE 1. Enterotoxic and lethal activities of Y. bercovieri CSF in infant mice

<table>
<thead>
<tr>
<th>Source</th>
<th>No. of strains producing lethal CSF (% lethality)</th>
<th>No. of strains (mean fluid accumulation ratio ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Clinical</td>
<td>52 (69)</td>
<td>56 (0.106 ± 0.02)</td>
</tr>
<tr>
<td>Foods</td>
<td>5 (100)</td>
<td>5 (0.105 ± 0.0)</td>
</tr>
<tr>
<td>Other</td>
<td>7 (64)</td>
<td>8 (0.102 ± 0.002)</td>
</tr>
<tr>
<td>Total</td>
<td>64 (69)</td>
<td>69</td>
</tr>
</tbody>
</table>

* The percentages shown for clinical, food, and other isolates are expressed as the percent of lethal CSF-producing strains among the strains obtained from each source (e.g., 69% of the 75 clinical isolates yielded lethal CSF). The percentage shown for total isolates is for the CSF of all 93 Y. bercovieri strains tested.

b Includes strains isolated from animals and environmental samples and those of unknown origin.

submucosa (Fig. 2). These histological observations are similar to those observed after exposure to known heat-stable enterotoxins, such as Y. enterocolitica YST I and Escherichia coli ST (7).

The ability of various concentrations of ammonium sulfate (25, 50, 75, and 90% saturated, final concentration) to precipitate the enterotoxin in the CSF was examined in order to determine (i) whether the toxin could be precipitated by ammonium sulfate, as are other proteins, and (ii) the lowest concentration which would precipitate >90% of the toxin. Seventy-five percent saturated ammonium sulfate yielded optimal amounts of toxin, and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis analysis of the CSF and the precipitated toxin preparation showed that some contaminating proteins were removed by the precipitation step (data not shown). In addition, the precipitation step removed large amounts of nonprotein contaminants (e.g., media pigments) from the toxin preparation. The precipitate was dissolved in and dialyzed against 50 mM ammonium bicarbonate (volatile buffer) and was freeze-dried. The enterotoxic activities of this preparation (designated crude toxin preparation) and of the CSF were heat stable; i.e., they remained active after boiling for 15 minutes. Thus, the toxin was tentatively called the Y. bercovieri heat-stable enterotoxin (YbST). As with YST I (3), YbST activity was not affected by incubation (37°C; 2 h) with trypsin, chymotrypsin, pepsin, papain, DNase, and RNase (all at 20 to 40 μg/ml). Activity was lost after incubating (37°C; 15 min) the crude preparation with a reducing agent (3 mM dithiothreitol), which suggests that disulfide bonds may be critical for the biological activity of YbST, and after boiling in the presence of a denaturing agent (0.1% SDS) for 3 min. Testing for pH stability was done by incubating (37°C; 4 h) samples of crude toxin at pH 11, pH 3.5, and pH 2, readjusting the pH to 8.5, and testing the preparations in infant mice. No reduction in enterotoxic activity was observed for any of the pH conditions tested, unlike observations with E. coli ST, which is resistant to low pH but is destroyed by exposure to pH 11 for 4 h at 37°C (11), but similar to observations with Y. enterocolitica YST I, which is unaffected by pH 1 through 11 (3).

Neutralization experiments with antisera raised against YbST and YST I indicated that the two toxins are immunogenically distinct from one another. The fluid accumulation responses normally elicited by the toxins were prevented by incubation with the homologous antisera but not by incubation with the heterologous antisera or with normal, preimmunization sera (Table 2). In addition, the lethal activity of CSF containing YbST was neutralized by the anti-YbST antiserum but not by the antiYST I antiserum or by normal serum.

DNA hybridization studies confirmed that YbST is different from YST I and showed that it is distinct from YST II. The experiments were performed essentially as described earlier (14) under both high-stringency conditions (incubation in 3× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] at 65°C and a final wash in 2× SSC at 68°C) and low-stringency conditions (incubation in 6× SSC at 42°C and a final wash in the same buffer at 56°C). Hybridized filters were exposed (~70°C, 24 to 48 h) to X-Omat RP film in a cassette containing enhancing screens, and bound label was visualized by developing the film in an automatic film developer. Colony blots of the 93 Y. bercovieri strains in our collection did not show hybridization with probes for the genes encoding the two reported (13, 15) Y. enterocolitica enterotoxins (YST I and YST II). The yst I probe has been described previously (17); the yst II gene has not yet been fully cloned and sequenced, but an experimental probe was kindly provided by Donald Robertson, University of Idaho, Moscow. Appropriate positive controls (Y. enterocolitica 8081 and 937, which are YST I- and YST II-producing strains, respectively) were included in all experi-

FIG. 1. Small intestines of infant mice 2.5 h after oral administration of enterotoxin-containing Y. bercovieri CSF (left) and TSB/YE (right).

FIG. 2. Light microscopy of the small intestines of infant mice 2.5 h after oral administration of TSB/YE (top; magnification, ×100) and enterotoxin-containing Y. bercovieri CSF (bottom; magnification, ×40).
TABLE 2. Effect of homologous and heterologous antisera on Ybst- and YST I-induced fluid accumulation in infant mice

<table>
<thead>
<tr>
<th>Treated preparations</th>
<th>Fluid accumulation ratio after treatment with:</th>
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<tbody>
<tr>
<td></td>
<td>TSB/YE</td>
</tr>
<tr>
<td>Ybst</td>
<td>0.130 ± 0.01</td>
</tr>
<tr>
<td>YST I</td>
<td>0.087 ± 0.02</td>
</tr>
<tr>
<td>TSB/YE</td>
<td>0.047 ± 0.01</td>
</tr>
</tbody>
</table>

a Aliquot (600 μl) of Ybst- and YST I-containing CSF were incubated (1 h; 37°C) with an equal volume of undiluted homologous and heterologous antisera, TSB/YE, and normal sera prior to assaying in infant mice.

b All fluid accumulation ratios are means (minimum of six animals) ± standard deviations.

c A gift from Donald Robertson, University of Idaho.

d Antiserum against Ybst was raised in 30 female, adult (25- to 30-g), CD-1 albino mice (Charles River Laboratories, Inc., Wilmington, Mass.) by subcutaneous administration of a water-in-oil emulsion (0.2 ml) composed of equal parts of boiled Y. bercovieri 6519 CSF (concentrated ca. 100-fold by ultrafiltration with a 3-kDa cut-off membrane) and Freund incomplete adjuvant. The mice received injections on day 0 and at weeks 5, 9, and 11 and were exsanguinated 1 week after the last injection. The sera were pooled, heat treated (56°C; 30 min), and stored at 4°C.

d ND, not done.

ments, and they gave the expected strong hybridization signals with the appropriate yst I and yst II probes. The colony blot hybridization results obtained with the yst II probe are shown in Fig. 3.

Subsequent studies to determine if a known, non-Yersinia enterotoxin enterotoxin was responsible for the biological activity associated with Y. bercovieri CSF involved screening colony blots of the 93 Y. bercovieri strains in our collection with fragment probes derived from known enterotoxin genes (E. coli LT, ST6, ST8, ST19, SLT I, SLT II, and EAST I). Hybridization conditions were as described above. None of the Y. bercovieri strains hybridized (under high- or low-stringency conditions) with the above probes (data not shown), which further supports the idea that Ybst is distinct from other known enterotoxins. Y. bercovieri strains also failed to hybridize with other known Yersinia virulence markers, such as probes derived from Y. enterocolitica virulence plasmid or from inv and ail chromosomal genes (the genetic probes and screening conditions have been described previously [14]).

In view of the apparent novelty of the enterotoxin produced by Y. bercovieri, the enterotoxin was characterized further. Examination of the kinetics of Ybst production by Y. bercovieri 6519 grown at 4, 28, and 37°C in TSB/YE showed that maximal amounts of toxin activity were obtained after 24 to 48 h of incubation at 28°C. Detectable levels of toxin were observed in the 4°C cultures only after prolonged (144- to 168-h) incubation; production of Ybst at 37°C also was limited, with borderline positive results for CSF obtained after 96 h of incubation. Maximal amounts of YST I activity in CSF of TSB/YE-grown Y. enterocolitica also have been observed when the cultures were grown at 28°C for 24 to 48 h (3).

Size exclusion chromatography of the crude toxin preparation with Sephadex G-50 fine (Pharmacia Biotech, Piscataway, N.J.) indicated that Ybst has an apparent molecular weight (MW) of about 12,000 (as determined after calibrating the column with globular proteins of known MW). However, because of the results of published studies on other heat-stable enterotoxins (15), it is possible that Ybst forms aggregates or binds to medium components, and, therefore, the actual MW of the toxin may be lower than that estimated by size exclusion chromatography. A more accurate estimation of the size of Ybst should be possible after Ybst is purified to homogeneity.

The isoelectric point of Ybst was estimated by high-speed isoelectric focusing (21) of a crude toxin preparation at 4°C, in a sucrose density gradient (pH 2.5 to 10) containing 1% Ampholine buffer (Pharmacia Biotech) and glycine. The pH of each fraction (4 ml) was determined at 4°C, and the fractions were dialyzed overnight at 4°C against 10 mM Tris-hydrochloride buffer (pH 7.6) and tested for enterotoxic activity in infant mice. Three fractions in the pH range 2.8 to 4.0 killed the mice and caused typical fluid accumulation in the intestines; all other fractions were negative. To obtain a more precise isoelectric point, experiments were performed using narrow pH range (pH 2.5 to 4), horizontal electrofocusing with the PhastSystem apparatus (Pharmacia Biotech). Gel slices were extracted with Tris-HCl buffer, and the solutions were tested for enterotoxic activity. The peak of activity was detected in the range of pH 3.5 to 3.8.

Using chambers were used (9) in order to determine whether Ybst-elicited fluid secretion is associated with changes in electrical current in intestinal mucosa. Toxin samples (Ybst-containing CSF and crude toxin preparation) were tested in four separate experiments. The potential difference was measured, and short circuit current (Isc) and tissue resistance were calculated. Variation of potential difference (Isc) and tissue re-

FIG. 3. Colony blot hybridization of 34 Y. bercovieri strains with the yst II probe. Numbers: 1 through 34, Y. bercovieri strains; 35 through 38, E. coli strains producing LT, STp, STg (35 and 36), SLT I (37), and SLT II (38); 39 and 40, YST I-producing Y. enterocolitica strains A2635 and 8081, respectively; 41, 43, and 44, YST II-producing Y. enterocolitica strain 937; 42, no bacteria. The negative results obtained with 59 additional strains of Y. bercovieri were identical to those shown in this figure (data not shown).
Y. bercovieri activity (shown as an increase in $I_{sc}$) reached a plateau (ca. 42 $\mu$A/cm²) at approximately 100 min of incubation and was reversible, i.e., returned to normal after removal of the enterotoxin (Fig. 4). The $I_{sc}$ values obtained with the two YbST preparations were comparable to those we saw with E. coli ST$_{0}$ (40 $\mu$A/cm²), YST I (38 $\mu$A/cm²), and E. coli EAST (40 $\mu$A/cm²). The mechanism by which YbST increases the $I_{sc}$ of intestinal mucosa (and by which it elicits the observed massive fluid secretion in the small intestines) remains to be determined.

In summary, we have discovered and partially characterized a novel, heat-stable enterotoxin produced by Y. bercovieri, a recently identified Y. enterocolitica-like species. Although YbST shares a number of characteristics with other heat-stable enterotoxins (heat stability, stability to a variety of hydrolytic enzymes and to a wide range of pH, apparent molecular size, and activity in infant mice and in Ussing chambers), it appears to be a different enterotoxin. For example, (i) YbST-producing strains do not hybridize with genetic probes for other enterotoxins, (ii) YST I-neutralizing antiserum does not neutralize YbST activity, (iii) YbST-neutralizing antiserum does not neutralize YST I activity, and (iii) massive fluid secretion caused by YbST-containing CSF and by partially purified YbST is associated with the death of infant mice. Based on the results of our screening of Y. bercovieri strains for known Yersinia virulence markers and for genes encoding some known enterotoxins, YbST is the only putative virulence factor identified so far in Y. bercovieri. Further studies are needed to determine whether it plays a role in the pathogenesis of diarrheal disease associated with Y. bercovieri infection.

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REFERENCES

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