Growth of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* Biotype 3B Serotype O3 Inhibited on Cefsulodin-Irgasan-Novobiocin Agar

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A total of 169 strains of *Yersinia* spp. were analyzed for their ability to grow on two different kinds of cefsulodin-Irgasan-novobiocin (CIN) agar containing 15 or 4 μg of cefsulodin per ml, on salmonella-shigella agar, and on MacConkey agar. CIN media inhibited the growth of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* biotype 3B serotype O3 (3B/O3) but not the growth of the other *Yersinia* organisms used. Relative to growth on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) with 6% yeast extract, 48 and 44% of *Y. pseudotuberculosis* and *Y. enterocolitica* 3B/O3 strains, respectively, were inhibited on CIN I agar (low cefsulodin concentration), and 83 and 54%, respectively, were inhibited on CIN II agar (high cefsulodin concentration) after incubation for 24 h at 32°C. The inhibition of *Y. pseudotuberculosis* growth was significantly more extensive on CIN II agar than on CIN I agar. The MICs of cefsulodin and novobiocin clearly indicated a higher susceptibility for *Y. pseudotuberculosis* than for the other *Yersinia* organisms at 32°C. All *Y. pseudotuberculosis* strains were susceptible to cefsulodin at 15 μg/ml (the approximate concentration used in CIN II agar). *Y. enterocolitica* 3B/O3 strains were resistant to cefsulodin, Irgasan, and novobiocin at the concentrations used in CIN media. These findings show that cefsulodin inhibits the growth of *Y. pseudotuberculosis* at the concentration used in CIN media and that growth inhibition of *Y. enterocolitica* 3B/O3 is related to a component of the CIN Base.

Different agar plating media have been used to isolate *Yersinia* organisms from various specimens (5, 6, 13). Schiemann (10) developed a new selective agar medium, cefsulodin (4 or 15 mg/liter)-Irgasan (4 mg/liter)-novobiocin (2.5 mg/liter) (CIN) agar, specifically for the isolation of *Yersinia enterocolitica*. Many investigators have reported that CIN agar was the most effective among selective differential plating media for the recovery of *Y. enterocolitica* from clinical specimens (6), animals (1, 17), food (5), and water (15). Weber et al. (17) reported that CIN agar was not only effective for the isolation of *Y. enterocolitica* but was also effective for the isolation of *Yersinia pseudotuberculosis* from the tonsils of pigs. Two different kinds of CIN agar, containing 4 mg (*Yersinia* selective agar; Difco Laboratories, Detroit, Mich.; hereafter designated CIN I) or 15 mg (*Yersinia* selective agar; Oxoid Ltd., London, England; hereafter designated CIN II) of cefsulodin per liter, are commercially available. CIN agar is used for the isolation of *Y. enterocolitica* and *Y. pseudotuberculosis* from various specimens (9, 12–14, 16). Although CIN agar appears to be a promising selective medium, Kaneko et al. (7) reported that colonies of *Y. pseudotuberculosis* incubated at 32°C for 24 h on CIN II agar were 0.1 to 0.5 mm in diameter and were smaller than those of other *Yersinia* organisms. Moreover, we found that CIN II agar has not been adequately evaluated for the recovery of *Y. enterocolitica* biotype 3B serotype O3 (3B/O3) and *Y. pseudotuberculosis* from clinical specimens and meat. The purpose of the present study was to determine whether *Yersinia* strains would regularly grow on CIN I and CIN II agars.

**MATERIALS AND METHODS**

**Test organisms.** A total of 169 strains of *Yersinia* spp. (46 *Y. enterocolitica* 3B/O3 strains, 53 *Y. enterocolitica* biotype 4 serotype O3 (4/O3) strains, 5 *Y. enterocolitica* biotype 2 serotype O5,27 (2/O5,27) strains, 2 *Y. enterocolitica* biotype 2 serotype O9 (2/O9) strains, 1 *Y. enterocolitica* biotype 1 serotype O8 (1/O8) strain, 1 *Y. enterocolitica* biotype 1 strain, 58 *Y. pseudotuberculosis* strains, 1 *Yersinia frederiksenii* strain, 1 *Yersinia intermedia* strain, and 1 *Yersinia kristensenii* strain) were used in this study. Of these, 61 were from our stock culture collection (46 3B/O3 strains isolated from 24 humans, 15 pigs, 3 dogs, 3 pork samples, and river water; 53 4/O3 strains from 22 humans, 22 pigs, 7 dogs, and 2 pork samples; 5 2/O5,27 strains from 1 human and 4 pigs; 12/09 strain from a human; 1 strain each of *Y. enterocolitica* biotype 1, *Y. frederiksenii*, *Y. intermedia*, and *Y. kristensenii* isolated from pork; 1/08 strain from a human was provided by T. Maruyama (Tokyo Metropolitan Research Laboratory of Public Health, Tokyo, Japan), and 1 2/09 strain from a human and 58 *Y. pseudotuberculosis* strains belonging to serotypes la, 1b, 2a, 2b, 2c, 3, 4a, 4b, 5a, 5b, and 6 (5 strains each), 7 (2 strains), and 8 (1 strain) and isolated from 33 humans, 4 monkeys, 1 dog, 1 raccoon, 1 hare, 6 mice, 4 guinea pigs, 1 squirrel, 3 pigeons, 1 duckling, 1 water sample, and 2 unknown sources were gifts from M. Tsubokura (Department of Veterinary Microbiology, Faculty of Agriculture, Tottori University, Tottori, Japan).

**Stool samples.** Stools from 37 patients infected with *Y. enterocolitica* 3B/O3 (16 patients), *Y. enterocolitica* 4/O3 (11 patients), and *Y. pseudotuberculosis* (10 patients) were used. These samples were stored in Cary-Blair transport medium.

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(BBL Microbiology Systems, Cockeysville, Md.) at 4°C and were examined within 1 week after sampling.

**Media.** CIN I agar, CIN II agar, and *Yersinia* selective agar base (CIN II Base; Oxoid) were used in addition to MacConkey (MAC; Nissui Pharmaceutical Co., Tokyo, Japan) agar, salmonella-shigella (SS; Nissui) agar, and Trypticase soy agar (BBL) with 6% yeast extract (Difco) (TSY).

**Comparison of selective media for recovery of *Yersinia* spp. from clinical specimens.** The abilities of MAC, CIN I, and CIN II agars to support the growth of *Y. enterocolitica* serotype O3 and *Y. pseudotuberculosis* were determined. Stools were diluted with 0.067 M phosphate buffer solution (pH 7.6), and 0.1 ml of each dilution was plated on MAC, CIN I, and CIN II agars. The MAC plates were incubated at 25°C for 48 h, and the CIN I and CIN II plates were incubated at 32°C for 24 h. The growth inhibition ratio (\(\log_{10}(n_{MAC}/n_{CIN})\)) for these organisms was expressed as a difference in \(\log_{10}\) CFU between growth on MAC agar and growth on CIN I and CIN II agars. Isolation and identification of *Yersinia* spp. were done as described previously (2).

**Evaluation of selective media.** The abilities of the CIN I, CIN II, CIN II Base, SS, and MAC media to support the growth of pathogenic *Y. enterocolitica* and *Y. pseudotuberculosis* and environmental *Yersinia* organisms were determined. Test strains were subcultured in Trypticase soy broth at 25°C for 48 h. The inocula for this study consisted of suspensions of a pure culture containing approximately \(10^8\) CFU/ml. Colony counts of the strains on each test medium were made by a modification of the drop technique of Miles et al. (8); 0.04 ml of each suspension diluted 10-fold from \(10^0\) to \(10^7\) with peptone broth was placed on each test medium. Moreover, the mixtures (1:1) of virulent *Yersinia* organisms with environmental *Yersinia* organisms were placed on CIN II agar at concentrations diluted 10-fold from \(10^1\) to \(10^7\). The plates were examined for colony characteristics and the number of colonies on each test medium at 24 and 48 h of incubation at 32°C. The recovery rate of *Yersinia* organisms was expressed as a percentage of the count obtained on TSY.

**Determination of MICs.** The MICs of cefsulodin (Takeda Chemical Industries Ltd.), Irgasan (CIBA-GEIGY AG.), and novobiocin (Sigma Chemical Co., St. Louis, Mo.) were determined by using the agar dilution test on Sensitivity Test Agar (modified Mueller-Hinton medium; Nissui). Incubation was performed at 32°C.

### TABLE 1. Recovery of *Y. enterocolitica* serotype O3 and *Y. pseudotuberculosis* from clinical specimens

<table>
<thead>
<tr>
<th>Virulent <em>Yersinia</em> organism infecting feces</th>
<th>Medium (no. of strains tested)</th>
<th>No. of strains showing growth inhibition (\log_{10}(n_{MAC}/n_{CIN})) of:</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Y. enterocolitica</em> O3</td>
<td>CIN I (10)</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>CIN II (16)</td>
<td>5</td>
</tr>
<tr>
<td><em>Y. enterocolitica</em> 3B/O3</td>
<td>CIN I (2)</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>CIN II (9)</td>
<td>3</td>
</tr>
<tr>
<td><em>Y. pseudotuberculosis</em></td>
<td>CIN I (6)</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>CIN II (10)</td>
<td>1</td>
</tr>
</tbody>
</table>

\(a\) MAC agar plates were incubated at 25°C for 48 h, and CIN agar plates were incubated at 32°C for 24 h.

\(b\) Counts of *Y. enterocolitica* serotype O3 and *Y. pseudotuberculosis* were \(10^6\) to \(10^8\) CFU/g of feces on MAC agar.

**RESULTS AND DISCUSSION**

We compared the relative growth of 169 strains of *Yersinia* spp. on two different commercially available CIN media (differing mainly in their cefsulodin content) and on SS agar, MAC agar, and TSY. We found that the CIN media inhibited the growth of *Y. pseudotuberculosis* and *Y. enterocolitica* 3B/O3 but not the growth of other *Yersinia* organisms. Moreover, in 11 of 37 stools from patients infected with *yersiniae*, the counts for *Y. pseudotuberculosis* and *Y. enterocolitica* 3B/O3 on CIN agar were significantly lower than those on MAC agar, but the counts for *Y. enterocolitica* 4/03 on CIN agar were the same as those on MAC agar (Table 1). Growth inhibition of 1 to 2 logs was observed with *Y. enterocolitica* 3B/O3 for 27% of the stools with CIN II agar. Growth inhibition of 2 to 6 logs was observed with *Y. pseudotuberculosis* for 80% of the stools with CIN II agar, and inhibition of 2 to 3 logs was observed for 50% of the stools with CIN I agar.

In the evaluation studies of selective media, the recovery rate of *Y. enterocolitica*, *Y. pseudotuberculosis*, *Y. pseudotuberculosis* biotype 1, *Y. frederikseni*, *Y. intermedia*, and *Y. kristensenii*. One strain failed to grow on this medium.

### TABLE 2. Ability of SS, MAC, and CIN media to support growth of *Yersinia* strains

<table>
<thead>
<tr>
<th>Medium</th>
<th>% Recovery at 24 h (48 h) of:</th>
<th><em>Y. pseudotuberculosis</em> (biotype/serotype)</th>
<th>Environmental <em>Yersinia</em> spp (4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3B/O3 (46)</td>
<td>4B/03 (53)</td>
</tr>
<tr>
<td>SS</td>
<td>72</td>
<td>104</td>
<td>99</td>
</tr>
<tr>
<td>MAC</td>
<td>107</td>
<td>104</td>
<td>104</td>
</tr>
<tr>
<td>CIN I</td>
<td>52 (91)</td>
<td>56 (92)</td>
<td>128 (128)</td>
</tr>
<tr>
<td>CIN II</td>
<td>17 (58)</td>
<td>46 (85)</td>
<td>95 (95)</td>
</tr>
<tr>
<td>CIN II Base</td>
<td>100 (101)</td>
<td>72 (99)</td>
<td>106 (106)</td>
</tr>
</tbody>
</table>

\(a\) The inoculum consisted of 0.04-ml suspensions of the test strains diluted to yield approximately \(10^8\) CFU on TSY. Colonies \(>0.5\) mm in diameter were counted on each medium. The recovery rate is expressed as a percentage of the mean count of colonies on TSY. SS and MAC agars were incubated at 25°C for 48 h; TSY and CIN agars were incubated at 32°C for 24 and 48 h.

\(b\) Figures in parentheses indicate the numbers of strains tested.

\(c\) *Y. enterocolitica* biotype 1, *Y. frederikseni*, *Y. intermedia*, and *Y. kristensenii*.

\(d\) One strain failed to grow on this medium.
frederiksenii, Y. intermedia, and Y. kristensenii ranged from 17 to 153%, depending on the species, serotype, and medium used (Table 2). All strains, except Y. pseudotuberculosis and Y. enterocolitica 3B/03 on CIN I and CIN II agars, grew well on SS, MAC, CIN I, CIN II, and CIN II Base media, and the overall maximum recovery ranged from 66 to 153%. CIN II agar was the least sensitive medium for Y. pseudotuberculosis and Y. enterocolitica 3B/03, with a recovery rate of 17 and 46%, respectively, at 24 h of incubation and 58 and 85%, respectively, at 48 h of incubation. CIN I agar was the second most inhibitory medium for growth of Y. pseudotuberculosis and Y. enterocolitica 3B/03, with a recovery rate of 52 and 56%, respectively, at 24 h of incubation and 91 and 92%, respectively, at 48 h of incubation. The CIN media inhibited the growth of Y. pseudotuberculosis and Y. enterocolitica 3B/03 but not the growth of other Yersinia organisms. The growth inhibition ratios ($n_{TSY}/n_{CIN}$) for Y. pseudotuberculosis and Y. enterocolitica 3B/03 on TSY and on CIN I, CIN II, and CIN Base media are shown in Fig. 1. Growth inhibition of 0.5 to 8 logs was observed for 19 Y. pseudotuberculosis strains on CIN II agar, as compared with 0.5 to 7 logs for 11 Y. pseudotuberculosis strains on CIN I agar; growth inhibition of Y. pseudotuberculosis was not observed with CIN II Base. One strain of Y. pseudotuberculosis failed to grow on CIN II agar; three colonies of this strain grew on CIN I agar. All strains except Y. pseudotuberculosis and Y. enterocolitica 3B/03 had colonies from 1.0 to 2.8 mm in diameter after 24 h of incubation. The growth of 55 and 73% of Y. pseudotuberculosis strains on CIN I and CIN II agars, respectively, appeared as pinpoint colonies (<0.4 mm in diameter) after 24 h of incubation. The diameter of Y. pseudotuberculosis colonies on CIN II Base, however, was mostly 0.5 to 0.9 mm.

The MICs for 58 Y. pseudotuberculosis strains clearly indicated a higher susceptibility to cefsludin and novobiocin for Y. pseudotuberculosis than for the other Yersinia organisms but that all strains tested were resistant to Irgasan and novobiocin at the concentrations used in CIN media at 32°C (Table 3), as was found for Y. enterocolitica by Schiemann (11). The MICs of cefsludin for Y. pseudotuberculosis were 6.25 to 25 ug/ml, 4 of 58 Y. pseudotuberculosis strains were susceptible to cefsludin at the concentration used in CIN II agar, and growth of the
remaining 54 strains was inhibited by cefsulodin at the approximate concentration used in CIN II agar (Table 3). The growth inhibition of Y. pseudotuberculosis was significantly more extensive with CIN II agar than with CIN I agar (which contains less cefsulodin). These findings demonstrate that cefsulodin inhibits the growth of Y. pseudotuberculosis at the concentration used in CIN media and suggest that novobiocin may exert some influence on the inhibition of growth of Y. pseudotuberculosis.

On the other hand, growth inhibition of 0.5 to 2 logs was observed with 11 to 21 Y. enterocolitica 3B/03 strains on CIN I, CIN II, and CIN II Base media. The growth of 76, 74, and 61% of the Y. enterocolitica 3B/03 strains on CIN I, CIN II, and CIN II Base media, respectively, appeared in the form of pinpoint colonies or a mixed culture of colonies from 0.5 to 1.6 mm in diameter or both; the pinpoint colonies were present at averages of 54, 47, and 35%, respectively. The MICs for Y. enterocolitica 3B/03 indicated that all strains tested were resistant to cefsulodin, Irgasan, and novobiocin at the concentrations used in CIN media at 32°C (Table 3). This phenomenon indicated that growth inhibition of Y. enterocolitica 3B/03 is related to a component of the CIN Base and not to antibiotics present in the CIN media. It remains to be clarified whether the relative sensitivity of Y. enterocolitica 3B/03 strains to CIN might be geographically limited to Japan.

Head et al. (6) reported that a minor weakness of CIN media is that colonies of Citrobacter freundii, Serratia liquefaciens, and Enterobacter agglomerans may not be reliably differentiated from Y. enterocolitica. In our study, the colonies of Y. pseudotuberculosis and Y. enterocolitica serotype O3 strains on CIN media had a deep-red center with a sharp border surrounded by a translucent zone; the center color, however, was deeper on CIN I agar than on CIN II agar. The center color of Y. enterocolitica serotypes O5,27, O8, and O9 resembled that of the environmental Yersinia organisms, especially Y. intermedia, on CIN II agar more than on CIN I agar. With 48 h of incubation, the color of the colonies of the virulent Yersinia strains faded, and Y. enterocolitica serotypes O5,27, O8, and O9 were indistinguishable from environmental Yersinia organisms, especially in mixed cultures with Y. intermedia.

We used SS agar simultaneously with MAC agar until 1983 and then used CIN II agar simultaneously with MAC agar for the isolation of Y. pseudotuberculosis and Y. enterocolitica from various specimens. The isolation frequency of Y. pseudotuberculosis and Y. enterocolitica serotype O3 from patients showed a tendency toward increase during our recent 7-year study in Japan (4). In particular, of Y. enterocolitica serotype O3 strains, the new biopsy 3B phage type 2 strain was recovered from clinical samples, pig samples, and pork with a tendency toward increase since 1972 (3). However, our results with clinical specimens (Table 1) and pure cultures (Table 2 and Fig. 1) demonstrated that most of the strains of Y. pseudotuberculosis and Y. enterocolitica 3B/03 showed growth inhibition when exposed to CIN media. Although CIN medium alone is sometimes used for the isolation of Yersinia organisms from various specimens, simultaneous testing of various specimens (especially meat samples highly contaminated with environmental Yersinia organisms) with other selective media and incubation for an additional 24 h should be done to obtain accurate results.

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LITERATURE CITED


