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Partial Characterization of *Yersinia intermedia* Bacteriocin

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Yersinia intermedia produces a temperature-dependent (25°C) bactericidal substance that is active against other *Yersinia* species. Crude preparations of the inhibitory substance were inactivated by chymotrypsin, trypsin, pronase, and heating but were not affected by lipolytic enzymes, chloroform, or other organic solvents. These data suggest that the active molecule is a bacteriocin of a proteinaceous nature. The molecular weight of the bacteriocin was estimated to be >14,000. Exposure of agar fragments containing the active component to a pH range of 3 to 11 did not affect bactericidal activity. Bactericidal activity against the *Y. frederiksenii* indicator strain was shown by simultaneous and deferred antagonism and by the associative culture technique. The liquor from cell-free macerated agar fragments and broth cultures, however, were devoid of antibacterial activity.

Bacteriocins are high-molecular-weight bactericidal substances that are produced by gram-positive and gram-negative bacteria and that are active against closely related bacteria (7). Among members of the genus *Yersinia*, bacteriocin-like activity has been reported for *Yersinia pestis* (1) and *Y. pseudotuberculosis* (3). Recently, we described a bacteriocin-like substance produced by *Y. intermedia* which exhibited temperature-dependent activity against *Y. enterocolitica*, *Y. frederiksenii*, *Y. intermedia*, and *Y. kristensenii*, but not against other members of the family *Enterobacteriaceae* (2). The bacteriocinogenic activity of *Y. intermedia* was demonstrated by the lawn-spotting technique after growth of the producer strain at 22 but not 37°C.

The earlier study from our laboratory (2) mainly dealt with the temperature-dependent expression and the spectrum of antimicrobial activity of the *Y. intermedia* bacteriocin. We present here data on the partial characterization of the active component of *Y. intermedia*.

MATERIALS AND METHODS

The bacteriocinogenic *Y. intermedia* test strain was one of seven previously described producer strains. It was isolated from clinical material submitted to the microbiology laboratory of The Mount Sinai Hospital, New York, N.Y. (2). The indicator strain, *Y. frederiksenii* (Y.e. 867), was obtained through the courtesy of Don Brenner, Centers for Disease Control, Atlanta, Ga. The test strains were maintained at room temperature and periodically subcultured onto Trypticase soy agar (TSA; BBL Microbiology Systems, Cockeysville, Md.).

Demonstration of bacteriocin activity. The protocol (Fig. 1) for assessing biological activity of the *Y. intermedia* bacteriocin was based on published techniques (2, 4, 7, 8). The bacteriocin activity of *Y. intermedia* against various indicator strains initially was assayed by the lawn-spotting technique (4). For the deferred antagonism method of demonstrating bacteriocin activity, duplicate plates of TSA were spot inoculated with the producer strain, *Y. intermedia*, and the plates were incubated for 18 h at 25 and 37°C (Fig. 1). After incubation, the growth from the agar surface was removed with a sterile cotton-tipped applicator and, as the bacteriocin was known to be resistant to chloroform, the agar surface was sterilized by exposure to chloroform vapor for 30 min. This was followed by aeration. Antagonistic activity of the preformed bacteriocin against the indicator strain, *Y. frederiksenii*, was then assessed by flooding the agar surface with semisolid (0.4%) TSA containing 0.05 ml of an 18-h-old Trypticase soy broth (TSB; BBL Microbiology Systems) culture of the indicator strain. Then, the plates were reincubated at 37°C and examined for zones of inhibition of the indicator strain around the spot inoculum.

Associate culture technique. Aliquots containing 10^4 organisms each of the producer, *Y. intermedia*, and the indicator, *Y. frederiksenii*, strains were simultaneously inoculated into seven duplicate sets of tubes containing 10 ml of TSB and incubated at 25°C (permissive temperature) and 37°C (nonpermissive temperature). At 1-h intervals for 6 h and at the end of 24 h, 1.0 ml of culture was removed from each tube, and viable counts were performed by plating 10-fold serial dilutions onto TSA containing 1.0% raffinose and phenol red indicator. Then, the plates were incubated at 37°C (nonpermissive temperature for bacteriocin production). The dilution enabled separation of colonies, thereby allowing for the differentiation of *Y. intermedia* colonies, which were surrounded by a zone

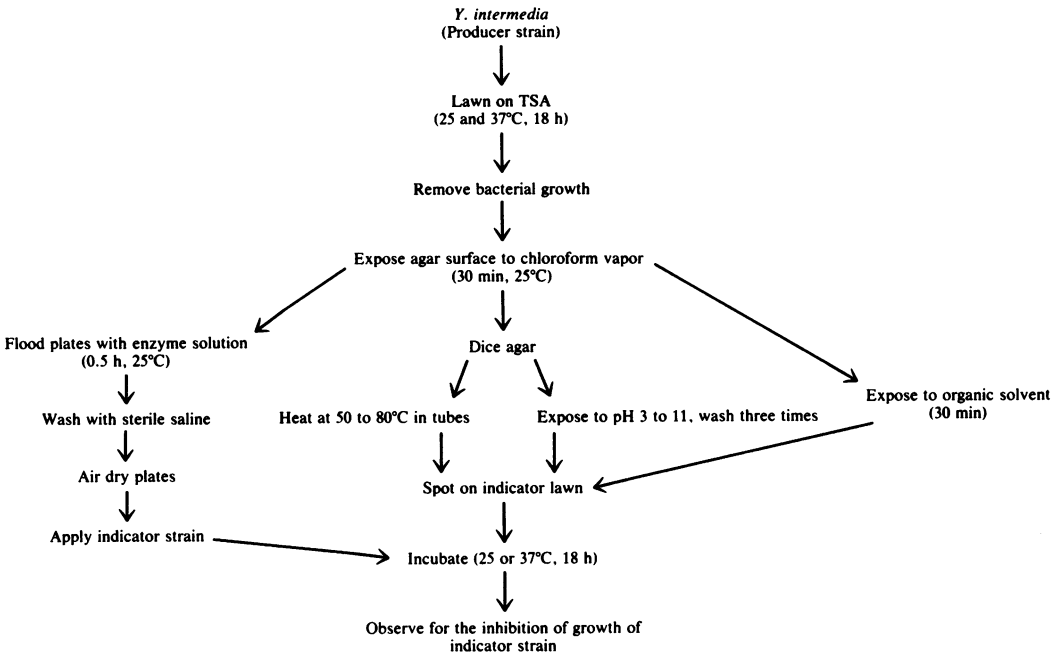

FIG. 1. Protocol for determining bacteriocin susceptibility to proteolytic enzymes and organic solvents and tolerance to temperature and pH fluctuations.

of yellow discoloration (raffinose fermentation), from *Y. frederiksenii* colonies, which were pink (raffinose negative). Colonies arising from dilutions plated onto sheep blood agar also were transferred to the raffinose agar medium by replica plating with sterile filter paper which was cut to fit the internal chamber of the petri dish.

Response to various enzymes and chemicals. The *Y. intermedia* bacteriocin was exposed to a variety of enzymes and chemical agents to define its nature. Exposure to trypsin, chymotrypsin, pronase, lipase, and phospholipases A_2, C, and D (Sigma Chemical Co., St. Louis, Mo.) was performed by flooding the sterile TSA medium containing preformed bacteriocin with the various enzyme solutions (Fig. 1). Susceptibility to reagent grade ethanol, methanol, acetone, and ethyl acetate was determined by exposing the agar surface to fumes of these solvents for 30 min. Untreated cultures served as controls for bacteriocin production.

To determine that the loss of bacteriocin activity after exposure to proteolytic enzymes and organic solvents was due to the inactivation of bacteriocin rather than to the destruction of bacteriocin receptor sites on the indicator strain by residual enzyme or solvent in the medium, the *Y. frederiksenii* indicator strain was also exposed to these agents for 5 to 30 min. Before testing, the treated *Y. frederiksenii* suspension was centrifuged. Then, the cells were washed twice with physiological saline, diluted to the original volume, inoculated onto 0.4% TSA, and flooded over the agar surface containing the preformed bacteriocin. The plates then were incubated at 37°C and read after 24 h.

Kinetics of bacteriocin production. Ten individual TSA plates were spot inoculated with the producer strain, *Y. intermedia*, incubated at 25°C, and sterilized with chloroform up to 10 h after 1-h intervals of growth. After removal of the growth with a sterile cotton-tipped applicator, the agar surface was overlaid with the indicator strain and incubated at 37°C for 18 h. The onset of bacteriocin production was determined by noting the age of the producer strain culture when it caused a distinct zone of inhibition of the indicator strain.

Bacteriocin activity in liquid media. To determine if liquid media would support the production and activity of the *Y. intermedia* bacteriocin, we inoculated the producer strain into 25 ml of a variety of broth media. These included TSB, TSB with 0.4% agar, TSB with 1.0% yeast extract, TSB with 0.4% agar and 5% citrated sheep blood, brain heart infusion broth, and Mueller-Hinton broth. These were incubated for 18 h at 25°C. To sterilize the cultures, we added 0.5 ml of chloroform and inverted the culture tubes to ensure mixing. After aeration for 2 h, an assay for the presence of the antagonistic factor was carried out by spotting 0.1 ml of the culture onto a lawn of the indicator strain, *Y. frederiksenii*. Controls consisted of spotting untreated suspensions in a similar way.

pH and thermal stability. To test the pH and thermal stability of the bacteriocin, we inoculated the entire surface of each TSA plate with the producer strain. After incubation for 18 h at 25°C, the growth was removed, and the agar surface was exposed to chloroform fumes. After aeration, the agar was removed and diced, and the agar pieces were added to 3 ml of buffer solution (pH range, 3 to 11). Separate minces were placed into glass vessels and exposed to temperatures of 50 to 100°C for various time intervals (Fig. 1). After

TABLE 1. Effect of enzymes, organic solvents, temperature, and pH on the activity of *Y. intermedia* bacteriocin

Agent or condition	Bacteriocin response
Trypsin (200 μg/ml, 25°C, 30 min)	Inactivation
Protease (100 μg/ml, 25°C, 30 min)	Inactivation
Chymotrypsin (100 μg/ml, 25°C, 30 min)	Inactivation
Lipase (50 μg/ml, 25°C, 30 min)	Resistance
Phospholipase	
A_2 (10 U/ml, 25°C, 30 min)	Resistance
C (4 U/ml, 25°C, 30 min)	Resistance
D (10 U/ml, 25°C, 30 min)	Resistance
pH	
3 (25°C, 30 min)	Resistance
11 (25°C, 30 min)	Resistance
Acetone	Resistance
Chloroform	Resistance
Ethanol	Resistance
Ethyl acetate	Resistance
Methanol	Resistance
Temperature (°C)	
55 (18 h)	Resistance
60 (18 h)	Resistance
65 (15 h)	Inactivation
70 (1 h)	Inactivation
80 (0.5 h)	Inactivation
100 (5 min)	Inactivation

the respective treatments, the agar slices were assessed for bacteriocin activity by placing the fragments on lawns of the indicator strain and examining for zones of inhibition after incubation for 18 h at 37°C. Agar fragments exposed only to chloroform fumes and buffer solutions (pH 7.2) served as controls.

Estimation of molecular weight of *Y. intermedia* bacteriocin. Estimation of the bacteriocin size (molecular weight) was made by placing dialysis membranes of various retention porosities (molecular weight, 1,000 to 14,000) onto the surface of the TSA medium. These were overlaid with the *Y. intermedia* producer strain which was suspended in 1.0% heated and cooled TSA. After 24 to 48 h of growth at 25°C, the membranes were removed, and the cell-free underlying medium was flooded with the indicator strain. After subsequent incubation for 18 h at 37°C, the plates were examined for zones of growth inhibition of the indicator strain. Controls consisted of growing the producer strain on a sterile membrane filter (0.45 μm; Millipore Corp., Bedford, Mass.) known to permit bacteriocin passage.

RESULTS

Associative culture. A bactericidal effect on the indicator strain, *Y. frederiksenii*, was demonstrated by the associative culture technique. No *Y. frederiksenii* colonies could be discerned on the raffinose agar medium by direct or replica plating after 4 h of cocultivation with the *Y. intermedia* producer strain, which was incubated at 25°C. In contrast, colony counts of 10^7 *Y. frederiksenii* per ml were obtained from subcultures of the associative cultures incubated at 37°C, a nonpermissive temperature for bacteriocin production.

Effect of physical and chemical agents on bacteriocin stability. Trypsin, chymotrypsin, and pronase were capable of inactivating the *Y. intermedia* bacteriocin, whereas lipase and phospholipases A_2, C, and D had no effect (Table 1). Incubation of the indicator strain, *Y. frederiksenii*, with proteolytic and lipolytic enzymes before testing did not alter its susceptibility to the *Y. intermedia* bacteriocin. These data reinforce the conclusion that the inhibitory action of trypsin, pronase, and chymotrypsin was on the bacteriocin and not on the indicator strain surface structure(s) that is required for bacteriocin binding. Exposure of agar surfaces containing preformed *Y. intermedia* bacteriocin to organic solvents (acetone, ethanol, ethyl acetate, and methanol) did not eradicate bacteriocin activity.

Bacteriocin production. The kinetics of *Y. intermedia* bacteriocin production in solid media are shown in Table 2. Zones of inhibition of the indicator strain, *Y. frederiksenii*, could be observed between 4 and 5 h after *Y. intermedia* growth initiation. Bacteriocin production continued throughout the growth cycle, with maximum production occurring after 14 to 16 h of growth, at which time the zones of inhibition of the indicator strain around the spot inoculum of *Y. intermedia* achieved their largest diameter (32 mm).

Detection of bacteriocin activity in liquid cultures. Attempts to demonstrate bacteriocin activity in chloroform-treated or filter-sterilized broth cultures were unsuccessful. Neither the addition of 0.4% agar as a stabilizing agent, yeast extract or sheep blood supplementation of TSB, nor growth in brain heart infusion broth or Mueller-Hinton broth resulted in the successful detection of the antibacterial component.

Effect of pH and temperature on bacteriocin stability. The *Y. intermedia* bacteriocin was found to be stable over a pH range of 3 to 11. Exposure of bacteriocin-containing agar slices to individual buffer solutions for 30 min did not

TABLE 2. Kinetics of *Y. intermedia* bacteriocin production

Age of Culture (h)	Zone of inhibition (mm) of *Y. frederiksenii* indicator strain
4	0
5	7
6	17
7	19
8	25
9	25
10–11	28
12–18	32

abolish bacteriocin activity, as these agar fragments were still encircled by zones of growth inhibition when superimposed on lawns of the indicator strain. The possibility that the bacteriocin was being protected from pH inactivation on the TSA minces was negligible, as these agar slivers were totally permeable to the surrounding buffer solutions (as indicated by methylene blue penetration in controls) during the 30-min exposure.

The thermal denaturation rate of the bacteriocin was determined by incubating the bacteriocin-containing agar slices at temperatures ranging from 50 to 100°C and superimposing them onto lawns of the indicator strain, *Y. frederiksenii*. By this technique, the antagonistic molecule was found to be stable at 55 to 65°C for up to 15 h, as seen by the zones of growth inhibition of the indicator strain around the agar slices obtained after exposure to heat. At 80°C, activity was lost in 30 min; activity ceased completely at 100°C after exposure for 5 min (Table 1). These latter data support the fact that heat permeates the agar slivers.

Molecular size. The antagonistic activity of the *Y. intermedia* bacteriocinogenic substance was shown after passage through the membrane filters. A large zone of inhibition was observed when the membrane was removed and the cell-free underlying agar was flooded with the *Y. frederiksenii* indicator strain. No zone of inhibition, however, could be seen after the producer strain was grown on a sterile dialysis membrane with a retention porosity of 14,000 daltons.

DISCUSSION

The inhibitory substance produced by *Y. intermedia* previously has been shown to display bacteriocin-like activity by its continued synthesis after repeated subculturing and its antibacterial spectrum, which is limited to closely related *Yersinia* species (2). Furthermore, analogous to many of the biological attributes of the yersiniae (6; G. Wauters, Ph.D. thesis, Vander, Louvain, Belgium, 1970), expression of the *Y. intermedia* active component is temperature dependent (2).

Comparison of the data gathered here for crude preparations containing the *Y. intermedia* inhibitory substance with data reported for other gram-negative and gram-positive bacteriocin-producing bacteria (5, 7, 8) strongly suggests that the *Y. intermedia* antibacterial component is mediated through a bacteriocin. Synthesis of the *Y. intermedia* active component during logarithmic growth and inactivation of its apparent proteinaceous nature by chymotrypsin, trypsin, pronase, and heating (100°C, 5 min) also support this conclusion.

Even though the *Y. intermedia* bacteriocin was active on the underlying cell-free agar surface after passage through a membrane filter, no activity was shown in crude preparations from various liquid media. Neither filtrates nor chloroform-sterilized supernatants showed antibacterial activity when spotted on lawns of the indicator strain. This could have occurred for several reasons. Bacteriocinogenic activity may be short-lived in the absence of a matrix for supporting affixation to an appropriate receptor site. Unexplained, however, is the failure of the addition of agar to the liquid medium to support bacteriocin activity, whereas provision of a solid agar (TSA) substrate achieved this desired result. Equally puzzling was the demonstration by the deferred antagonism method of the bacteriocinogenic component on cell-free agar, whereas the liquor, resulting from hand-macerated agar fragments containing the bacteriocin, was devoid of antibacterial activity.

It may be that *Y. intermedia* bacteriocin is closely associated with the cell surface of the producing cell and, after release from such a site, is rapidly inactivated, unless it is bound to the receptor site of the indicator cell. In the lawn-spotting or associative culture techniques, the articulation of bacteriocin with proximal susceptible indicator cells serves to stabilize the diffusing active component. In the absence of the indicator cells or a sufficient amount of agar, which both stabilize the bacteriocin, the bacteriocin apparently is inactivated.

The molecular weight of the *Y. intermedia* bacteriocin was >14,000. The finding that the bactericidal substance lacked a lipid component (as seen by resistance to lipolytic enzymes, chloroform, and other organic solvents) suggests that the bacteriocin may not be associated with the O antigen component, as has been documented for colicins (8). It may be, however, that the active moiety of the *Y. intermedia* bacteriocin dissociates from a larger complex molecule. It also may be that it exists in two or more molecular forms in equilibrium with the activity that exists in the proteinaceous component. Many of these questions will remain unresolved until the bacteriocinogenic substance can be recovered in its purified form.

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