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Production of Bacteriocin Inhibitory to *Listeria* Species by *Enterococcus hirae*

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A bovine intestinal bacterial isolate, identified as *Enterococcus hirae*, was found to produce a bacteriocin (designated hiraecin S) inhibitory to *Listeria monocytogenes* and other *Listeria* spp. Identification to species level was determined by comprehensive biochemical and morphological tests which were verified by DNA-DNA homology assays. The antimicrobial agent was inactivated by pronase and papain and was insensitive to catalase. The antimicrobial activity was not due to hydrogen peroxide or acid formation, nor was lysozyme or muramidase activity observed in cell-free bacteriocin preparations. Inhibition of selected gram-negative bacteria was not observed. Other enterococci were sensitive to the bacteriocin, and except for *Listeria* spp., no other gram-positive bacteria tested were inhibited.

Bacteriocin production is a common phenomenon among lactic acid bacteria (20, 40). In a survey of the enterococci (group D streptococci), Brock et al. (3) determined that more than 50 of 100 strains tested produced a bacteriocin. Galvez et al. (12) found that 40% of 90 human group D streptococci were capable of bacteriocin production. More recently, research in this field has centered on antimicrobial agents produced by food-grade organisms for use as preservatives in foods (14, 17, 39).

The genus *Enterococcus* has undergone much taxonomic revision. In 1984, the genus *Streptococcus* was split into *Enterococcus*, *Lactococcus*, and *Streptococcus* (34). The enterococci were composed of bacteria generally classified as group D fecal streptococci. This revision has led to a more accurate taxonomic grouping of the enterococci and has increased the number of distinguishable species assigned to this genus. On the basis of 16S rRNA sequences, at least 18 species in the *Enterococcus* genus have been described to date (4, 9, 27, 28, 32, 42). The conventional biochemical classification scheme of Facklam and Collins (9) was developed and verified by DNA-DNA hybridization. This system divides the enterococci into three groups which are further subdivided into species. This scheme was shown to be useful for biochemically characterizing enterococci. The species *Enterococcus hirae* was first described by Farrow and Collins (10). The initial isolates were obtained from swine intestines and chicken crops; subsequent isolates were from human sputum and cattle feces (10, 21).

Listeria monocytogenes is a psychrotrophic food-borne pathogen (31). Inhibition of *L. monocytogenes* and other *Listeria* spp. by enterococcal bacteriocins (1, 25, 29, 30) in addition to bacteriocins from a variety of other genera (6, 13, 14, 16, 24, 25, 39) has been reported. To date, no reports of bacteriocin production by *E. hirae* have been made. This report describes the isolation and characterization of a strain of *E. hirae*, obtained from a bovine, which produces a bacteriocin inhibitory towards *L. monocytogenes* and other *Listeria* spp. This bacteriocin was designated hiraecin S.

(Portions of this research were previously presented [35].)

MATERIALS AND METHODS

Bacterial isolation. Isolates of enterococci were obtained from enrichment cultures of bovine fecal samples as described previously (36). Briefly, feedlot cattle fecal grab samples were enriched in UVM-2 Listeria enrichment broth (BBL Microbiology Systems, Becton Dickinson, Cockeysville, Md.). Colonies surrounded by zones of inhibition on a background of *Listeria* spp. growth were isolated and purified to homogeneity by being streaked on TSBYE agar (tryptic soy broth agar plus 0.5% [wt/vol] yeast extract) and incubated at 37°C. Isolates were stored at −20°C in 75% (vol/vol) glycerol until used.

Bacteriocin assays. The agar well diffusion method of Schillinger and Lücke (33) was modified and used to assay antimicrobial activity. To neutralize the effects of bacterial acid production, 0.1 M MOPS (morpholinepropanesulfonic acid) buffer was incorporated into TSBYE agar and the pH was adjusted to 7.2 (TM agar). TM broth medium supported growth equivalent to that found in nonbuffered media but maintained the final culture pH at greater than 6.9 (data not shown). Target organisms, propagated in TM broth at 37°C for 16 h, were mixed in 8 ml of TM semisoft agar (0.75% [wt/vol]) to achieve a concentration of approximately 10^6 CFU/ml. This suspension was overlaid on a standard agar plate (15 by 100 mm) of TM agar and allowed to solidify at room temperature for 15 min. Six wells per plate (6-mm diameter) were made with a sterile cork borer. Ten microliters of 1:10-diluted antimicrobial agent-producing culture (whole-cell production assay) or cell-free antimicrobial test agent was added to each well. Plates were incubated for 18 to 24 h at 37°C, and zones of inhibition were measured to the nearest millimeter with a set of Vernier scale calipers with a dial fractional readout.

Phenotypic characterization. Isolates were initially characterized on the basis of the taxonomic scheme of Facklam and Collins (9). Carbohydrate fermentation tests were performed by the modified microtiter plate method (38), the Minitek method (BBL), and the tube method with both purple broth base and phenol red broth (Difco Laboratories, Detroit, Mich.) with 1% carbohydrates. Additional carbohydrate fermentation and enzymatic reactions were determined with

the API Rapid Strep system (API Analytab Products, Div. Sherwood Biomedical Industries, Plainview, N.Y.). Selected isolates were sent for specific analysis by the Vitek microbial identification system to outside laboratories, the U.S. Centers for Disease Control Streptococcal Reference Laboratory (Atlanta, Ga.) and the University of Nebraska Veterinary Diagnostic Laboratory (Lincoln, Nebr.). Lancefield group D antigen testing was performed with the Phadebact coagglutination test system (Karo Bio; Remel Inc., Lenexa, Kans.). Antibiotic sensitivity tests were performed according to the Kirby-Bauer disc sensitivity method (2).

DNA-DNA hybridization assays. To confirm the taxonomic position of the selected isolate, DNA homology assays were performed by the S1 nuclease method described by Johnson (19) as used by Knight et al. (22) and Knight and Shlaes (21). Briefly, DNA was isolated and purified from the test *Enterococcus* isolate by the Marmur method as described by Johnson (19). The purified DNA was tritium labeled by nick translation. ^{3}H-labeled thymidine was obtained from NEN DuPont (Bannockburn, Ill.). Nick translations were performed with a commercially available kit (Boehringer Mannheim GmbH, Mannheim, Germany). Cells from 600 ml of a tryptic soy broth (BBL) culture (17 h, 37°C) were treated with lysozyme and lysed with lysis buffer containing 20% sodium dodecyl sulfate. After repeated phenol-chloroform extractions, the isolated nucleic acid was RNase (Sigma Chemical Co., St. Louis, Mo.) treated, reextracted, and ethanol precipitated. An aliquot was quantitated by determining the A_{260}/A_{280} ratio. All samples were purified, so that this ratio was at least 1.6. DNA samples were held at −20°C before use.

All DNA was sheared by sonication (30 s, microprobe tip) and heat denatured (heated at 100°C for 5 min and then ice cooled) prior to use in the assay. Hybridizations were performed with a fivefold excess of nonlabeled DNA over tritium-labeled DNA for 24 h at 60°C. S1 nuclease (GIBCO-BRL, Bethesda, Md.) digestions were performed at 55°C for 1 h. S1 nuclease-digested samples were precipitated with 10% (vol/vol) trichloroacetic acid and collected on membrane filters. Filters were washed repeatedly with cold 5% trichloroacetic acid and allowed to air dry. [^{3}H]thymidine activity was determined by placing the dried membrane in a scintillation vial with 5 ml of Budget-Solve (Fisher Scientific, Pittsburgh, Pa.) and reading it in a Packard scintillation counter (Packard Instrument Co., Downers Grove, Ill.). Control hybridization reaction mixtures included labeled DNA hybridized with salmon sperm DNA and labeled DNA with homologous unlabeled DNA. Background counts from the salmon sperm DNA control were subtracted from all reaction results before percent homologies were calculated. The number of S1 nuclease-resistant counts per minute in the homologous reaction mixture was taken as 100% homology.

Partial bacteriocin purification. A partially purified bacteriocin preparation was made by ammonium sulfate precipitation. Antimicrobial agent-producing cultures were grown in 500-ml volumes of TM broth (see above) in Erlenmeyer flasks to late log or early stationary phase at 37°C with 100-rpm rotary agitation. Cells were pelleted by centrifugation, and the supernatant was decanted into a beaker set in an ice bucket. Solid ammonium sulfate was added stepwise over 60 min to achieve 75% saturation and allowed to stir for an additional 30 min on a magnetic stir plate. The precipitated suspension was centrifuged for 30 min at 10,000 × *g* at 5°C and decanted. The pellet was dissolved in MOPS-buffered saline (pH 7.2) to 5% of the starting volume. The concentrated solution was dialyzed against phosphate buffer (0.1 M; pH 7.2) with two changes of buffer at 5°C overnight. Dialyzed solution was filter sterilized, aliquoted, and frozen at −70°C.

Characterization of bacteriocin. The effect of protease treatment on the bacteriocin was tested. Pronase (0.8 U per well) (Calbiochem-Behring, La Jolla, Calif.) and papain (0.1 U per well) (Sigma Chemical Co.) dissolved in MOPS-buffered saline were added to separate wells containing the concentrated extract and assayed as described above. To test for inhibition due to hydrogen peroxide formation, catalase (Sigma Chemical Co.) was added to the semisoft overlay (163 U/ml of semisoft overlay) and assays were conducted as described. Lysozyme or muramidase activity in the concentrated antimicrobial agent from the isolate was assayed as previously described (5). Lyophilized cells of *Micrococcus lysodeikticus* (Sigma Chemical Co.) were used as a lysozyme substrate for the bacteriocin by monitoring the optical density at 450 nm at 25°C for up to 3 h. Hen egg white lysozyme was used as the control enzyme (Sigma Chemical Co.).

MIC. The relative sensitivity of *Listeria* spp. to the bacteriocin preparation was determined in a broth culture format. Target organisms were grown overnight in TM broth at 37°C and diluted in fresh TM broth to a cell density of 10^7 CFU/ml based on McFarland standards (Difco Laboratories). In a round-bottom microtiter plate, 100 μl of 3× TM broth was added to each well. The total protein content of the concentrated bacteriocin preparation was found to be 6.3 mg/ml as determined by the Pierce bicinchoninic acid assay (Pierce Chemical Co., Rockford, Ill.). The bacteriocin was serially diluted to 1:1,024. Both a positive control (no bacteriocin) and an uninoculated control were included. Each well received 100 μl of inoculum suspension and was incubated at 37°C for 8 h. After the incubation period, the A_{630} was determined in a BioTek 340I microplate reader (BioTek Instruments, Winooski, Vt.). The MIC was arbitrarily defined as the amount of total protein in the highest dilution of bacteriocin which inhibited growth in that well. This value was then divided by the absorbance of the inoculated control well (theoretical maximal growth) to normalize the effects of cell density.

RESULTS

Isolate identification. Phenotypic traits of the organism studied (code C311) are presented in Table 1. Genetic confirmation based on DNA relatedness (Table 2) showed that isolate C311 shared 75 and 86% homology with *E. hirae* ATCC 9790 and *E. hirae* ATCC 35220, respectively. Homology with the related species *Enterococcus durans*, *Enterococcus faecium*, and *Enterococcus faecalis* ranged from 0 to 6% in the assay system used. Organisms are considered to belong to the same species if they have 70% or more DNA homology under optimum hybridization conditions (10). On the basis of biochemical and morphological characteristics described above, the isolate C311 was identified as *E. hirae* as previously described (9, 10, 21, 34).

Spectrum of antimicrobial activity. Table 3 summarizes the antimicrobial activity spectrum of the bacteriocin produced by *E. hirae* C311. The bacteriocin was inhibitory to 13 of 15 *Listeria* spp. tested. These included an assortment of serotypes and sources. Two cattle isolates, *L. monocytogenes* C12-1-1-L and 81-2-2-L, were both sensitive. No measurable zone of inhibition was observed when *L. monocytogenes* V7 (serotype 1/2a) and *Listeria welshimeri* ATCC 49591 (petite

TABLE 1. Phenotypic characteristics of isolate C311

Parameter or test	Reaction or characteristic	Parameter or test	Reaction or characteristic
Morphology	Cocci	Urease	−
Gram reaction	+	Cellobiose	+
Group D antigen	+	Glucose	+
Sheep blood hemolysis	−	Esculin	+
Catalase	−	Glycerol	+
Pigmentation	−	Maltose	+
Capsule formation[a]	−	Melibiose	+
Motility	−	Raffinose	+
Growth			
At 45°C	+	Ribose	+
At 10°C	+	Salicin	+
At 4°C	+	Sucrose	+
At pH 9.6	+	Trehalose	+
In 40% bile	+	Fructose	+
In 6.5% NaCl	+	Arbutin	+
Tellurite	R[b]	α-Methyl-D-mannopyranoside	+
Tetrazolium reduction	+	Arabinose	−
Clindamycin (2 μg/disc)	R	Glycogen	−
Tobramycin (10 μg/disc)	R	Inulin	−
Novobiocin (0.1 μg/disc)	R	Lactose	−
Bacitracin (0.5 μg/disc)	R	Mannitol	−
Vancomycin (15 μg/ml)	S[b]	Melezitose	−
Litmus milk	A-SC[c]	Pullulan	−
Voges-Proskauer	+	Pyruvate	−
Arginine dihydrolase	+	Sorbitol	−
α-Galactosidase	+	Sorbose	−
Leucine arylamidase	+	Starch	−
Pyrrolidonylarylamidase	+	Xylose	−
Alkaline phosphatase	−	Rhamnose	−
β-Glucuronidase	−	Citric acid	−

[a] Determined by growth on TSBYE agar plus 5% (wt/vol) sucrose.
[b] R, resistant; S, sensitive.
[c] A-SC, acid-soft curd. The reaction was observed beginning at 6 days post inoculation.

phenotype) were tested in the well assay format. The parent strain of ATCC 49591, *L. welshimeri* ATCC 35897, was considerably more sensitive to the agent than was the petite derivative. Several bovine group D enterococci which were not identified to species level were inhibited by the bacteriocin (7 of 12 were sensitive). Except for these seven enterococci and the listeriae, no other gram-positive species tested were inhibited. No gram-negative species tested were sensitive to the effects of the agent. In the agar well assay format, colonies that were apparently resistant to the antimicrobial agent were occasionally observed within the zone of inhibition.

Table 4 lists the arbitrary MIC units of the bacteriocin for *Listeria* spp. *Listeria ivanovii*, *Listeria seeligeri*, and *Listeria murrayi* were the most sensitive species. *Listeria innocua* ATCC 33090 was found to be very sensitive to the antimicrobial agent when tested in the in situ assay, although the zone of inhibition was very small (Table 3), as was reflected in the MIC assay. *L. monocytogenes* V7 and *L. welshimeri* ATCC 49591 were inhibited by a high concentration of the concentrated bacteriocin, although no zone of inhibition was observed in the in situ well assay. On a total protein basis, 3 to 144 μg of total protein content was needed to inhibit the *Listeria* strains (Table 4). The antimicrobial agent of isolate C311 was not autoinhibitory.

TABLE 2. DNA relatedness between *Enterococcus* isolate C311 and other enterococci

Source of unlabeled DNA	% Homology with ^{3}H-labeled DNA from *Enterococcus* isolate C311[a]
E. hirae ATCC 9790	75
E. hirae ATCC 35220	86
E. durans ATCC 19432	6
E. faecalis ATCC 19433	2
E. faecium ATCC 19434	0–1

[a] Data are expressed as the means of at least two homology experiments conducted at 60°C.

Partial characterization of the bacteriocin hiraecin S. The concentrated bacteriocin from *E. hirae* C311 was found to be resistant to catalase addition, pH buffering, and heating at 100°C for up to 60 min and freeze-thaw resistant (Table 5). No lysozyme or lysozyme-like activity was detected. The addition of either pronase or papain with the bacteriocin in well assays eliminated any antimicrobial activity against *Listeria* spp. These data indicate that the antimicrobial agent is proteinaceous and that the inhibition is not due to the formation of hydrogen peroxide or acid or lysozyme activity.

DISCUSSION

Although other species of *Enterococcus* are known to produce bacteriocins which inhibit *Listeria* spp. (1, 24, 29, 30), this is the first known report of bacteriocin production by the species *E. hirae*. The bovine isolate studied in this work was identified as *E. hirae* on the basis of both phenotypic and DNA homology data. The phenotype expressed by the isolate is consistent with those reported by Farrow and

TABLE 3. Inhibitory spectrum of *E. hirae* C311

Target species	Strain (serotype)	Reaction to bacteriocin[a]	Assay type[b]
Listeria monocytogenes	Scott A (4b)	S	CF, WC
Listeria monocytogenes	V7 (1/2a)	R	CF, WC
Listeria monocytogenes	ATCC 15313 (4b)	S	WC
Listeria monocytogenes	ATCC 19113 (3)	S	WC
Listeria monocytogenes	C12-1-1-L (4)	S	WC
Listeria monocytogenes	81-2-2-L (4)	S	WC
Listeria monocytogenes	Brie 1 (1b)	S	WC
Listeria grayi	ATCC 19120	S	WC
Listeria innocua	ATCC 33090 (6a)	S	CF
Listeria innocua	LA-1	S	CF, WC
Listeria ivanovii	KC1714	S	CF, WC
Listeria murrayi	ATCC 25401	S	WC
Listeria seeligeri	ATCC 35967	S	CF, WC
Listeria welshimeri	ATCC 35897	S	CF, WC
Listeria welshimeri	ATCC 49591	R	CF, WC
Enterococcus faecalis	ATCC 19433	R	CF
Enterococcus hirae	ATCC 9790	R	CF
Enterococcus hirae	ATCC 35220	R	CF
Enterococcus hirae	C311	R	CF
Enterococcus spp.[c]	7 fecal strains	S	CF
Enterococcus spp.	5 fecal strains	R	CF
Lactobacillus casei	ATCC 393	R	CF
Micrococcus flavus	ATCC 10240	R	CF
Staphylococcus aureus	ATCC 25923	R	CF
Staphylococcus epidermidis	ATCC 12228	R	CF
Brochothrix thermosphacta	ATCC 11509[d]	R	CF
Escherichia coli	ATCC 25922	R	CF
Salmonella typhimurium	ATCC 14028	R	CF
Shigella flexneri	ATCC 12022	R	CF
Yersinia enterocolitica	ATCC 23715	R	CF

[a] S, sensitive; R, resistant.
[b] CF, assay of antimicrobial activity from cell-free bacteriocin preparation; WC, assay of antimicrobial activity from culture inoculated directly into agar well (see Materials and Methods).
[c] Bovine fecal isolates identified as group D *Enterococcus* spp.
[d] Assay conducted at 25°C.

Collins (10), Facklam and Collins (9), Knight and Shlaes (21), and Schleifer and Kilpper-Bälz (34) for *E. hirae*. Isolate C311 was resistant to both tobramycin and clindamycin. Knight and Shlaes (21) reported that isolates of *E. hirae* were resistant to both clindamycin and tobramycin while *E. durans* ATCC 19432 was sensitive to both antibiotics. Additionally, isolate C311 would acidify litmus milk but curd formation was observed to begin only after 6 days of incubation. *E. durans* ATCC 19432, on the other hand, acidified and clotted litmus milk in 24 h. Phenotypic identification was consistent with analysis of DNA homology between isolate C311 and two standard strains of *E. hirae*. Species closely related to *E. hirae*, including *E. faecalis*, *E. durans*, and *E. faecium* (39), showed little DNA homology as assayed. Other workers using this assay have reported a similar range of values for homology between *E. hirae* strains and *E. durans*, *E. faecium*, and *E. faecalis* (21, 22).

Konisky (23) defined a bacteriocin as a protein or protein complex synthesized by a bacterium which is inhibitory to a narrow range of other susceptible bacteria but is not self-inhibitory (autoinhibitory). By this definition, the antimicro-

TABLE 4. MIC of semipure *E. hirae* C311 bacteriocin for *Listeria* spp.

Species	Strain	Arbitrary MIC units[a]
L. seeligeri	ATCC 35967	22.2
L. murrayi	ATCC 25401	44.0
L. ivanovii	KC1714	74.3
L. welshimeri	ATCC 35897	298.1
L. grayi	ATCC 19120	395.7
L. monocytogenes	Scott A	614.5
L. monocytogenes	C12-1-1-L	666.9
L. monocytogenes	ATCC 19113	947.7
L. innocua	LA-1	1,259.2
L. monocytogenes	V7	5,928.0
L. welshimeri	ATCC 49591	6,700.0
L. innocua	ATCC 33090	7,581.6

[a] Two replicates of each MIC assay were performed. Values are expressed as the total protein content (in micrograms per milliliter) of the bacteriocin preparation in the well of the highest dilution with no growth after 8 h at 37°C. All values are normalized to the turbidity (A_{630}) of an inoculated control well containing no bacteriocin.

TABLE 5. Partial characterization of *E. hirae* C311 bacteriocin

Bacteriocin treatment	Inhibitory activity[a]
Catalase	+
MOPS buffer	+
Freeze-thaw	+
100°C for 60 min	+
Pronase	−
Papain	−

[a] Assays were conducted as described in Materials and Methods, with selected *Listeria* spp. as target organisms.

bial agent produced by *E. hirae* C311 is a bacteriocin. The significance of bacteriocin production in the organism's native environment is unclear, although it could likely offer a competitive advantage to the producing organism. Indeed, whether the bacteriocin is even produced in the bovine intestinal tract is not known. *E. hirae* has been isolated from the bovine intestinal tract with high frequency and appears to be a part of the normal enterococcal flora of several animals, including cattle, swine, poultry, horses, sheep, goats, rabbits, and dogs (7).

Enterococci are commonly isolated from foodstuffs (18, 29) as well as from animal intestinal tracts (7); however, the frequency of *E. hirae* associated with foods is not known. Bacteriocins from human isolates of *Streptococcus faecium* have been studied (24, 25). The workers reported inhibition of all serotypes of *Listeria*, other streptococci, *Clostridium perfringens*, and *Clostridium septicum*.

The observation of resistant colonies in the agar well assays leads to the conclusion that the target populations are composed of organisms with different sensitivities to hiraecin S. Mechanisms of bacteriocin resistance in *S. faecalis* are not defined, although plasmid mediation is reported (26). Strains of *Listeria* in this study showed a wide range of sensitivities to hiraecin S. Interestingly, the *L. welshimeri* test strains showed great differences in their sensitivities to hiraecin S but are closely related to one another. *L. welshimeri* ATCC 49591 is a naturally occurring petite-phenotype isolate derived from ATCC 35897 (37). This strain is constitutive for beta-glucosidase (esculinase) activity. The genetic basis and physiological parameters of bacteriocin production by *E. hirae* C311 are currently under examination.

Potential applications of this agent and/or organism remain to be studied. The use of bacteriocins as food preservatives has received widespread attention. This is especially true for bacteriocins derived from the microflora of fermented foods (14, 17) that are considered safe for human consumption. Enterococci are generally considered normal intestinal flora and not gastrointestinal pathogens. Under experimental conditions, however, *E. hirae* reportedly caused diarrhea in suckling rats less than 3 days old after they were orally inoculated with 3×10^8 organisms (8). *E. durans* has been reported to cause diarrhea in foals, gnotobiotic piglets, and infant rats (15, 41). Because of the intestinal origin of this isolate, its use as a probiotic would have to be carefully examined. *E. faecalis* and *E. faecium* are already used in this capacity (11). The significance of bacteriocin production by enterococci can now be further elucidated because of advances made in the taxonomy of this genus. While using potentially pathogenic organisms for food additives may be unlikely, bacteria such as *E. hirae* may serve as a source of genetic material for strain construction or as host-adapted probiotic cultures. The isolation of this strain expands the spectrum of known bacteriocin-producing species. The characteristics of hiraecin S and its relationship to other enterocins remain to be studied.

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