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Purification and Genetic Characterization of Enterocin I from *Enterococcus faecium* 6T1a, a Novel Antilisterial Plasmid-Encoded Bacteriocin Which Does Not Belong to the Pediocin Family of Bacteriocins

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Enterocin I (ENTI) is a novel bacteriocin produced by *Enterococcus faecium* 6T1a, a strain originally isolated from a Spanish-style green olive fermentation. The bacteriocin is active against many olive spoilage and food-borne gram-positive pathogenic bacteria, including clostridia, propionibacteria, and *Listeria monocytogenes*. ENTI was purified to homogeneity by ammonium sulfate precipitation, binding to an SP-Sepharose fast-flow column, and phenyl-Sepharose CL-4B and C_2/C_{18} reverse-phase chromatography. The purification procedure resulted in a final yield of 954% and a 170,000-fold increase in specific activity. The primary structure of ENTI was determined by amino acid and nucleotide sequencing. ENTI consists of 44 amino acids and does not show significant sequence similarity with any other previously described bacteriocin. Sequencing of the *entI* structural gene, which is located on the 23-kb plasmid pEF1 of *E. faecium* 6T1a, revealed the absence of a leader peptide at the N-terminal region of the gene product. A second open reading frame, ORF2, located downstream of *entI*, encodes a putative protein that is 72.7% identical to ENTI. *entI* and ORF2 appear to be cotranscribed, yielding an mRNA of ca. 0.35 kb. A gene encoding immunity to ENTI was not identified. However, curing experiments demonstrated that both enterocin production and immunity are conferred by pEF1.

Bacteriocins are bacterial proteins or peptides that inhibit strains and species that are usually, but not always, closely related to the producing bacteria (47). In recent years, several bacteriocins from gram-positive bacteria, in particular the lactic acid bacteria (LAB), have been identified and characterized (24, 30, 36). Some of these bacteriocins display fairly broad inhibitory spectra and have potential as food preservatives (13, 22, 24). Apart from the lantibiotic nisin, the most promising are those belonging to the pediocin family of bacteriocins (36). These bacteriocins are active against a broad spectrum of food spoilage and food-borne gram-positive pathogenic bacteria, including *Listeria monocytogenes*. Pediocin-like bacteriocins have been identified for various genera of LAB, including *Carnobacterium* (carnobacteriocins BM1 and B2 [42]), *Lactobacillus* (sakacins A [21] and P [48]), *Leuconostoc* (leucocin A [17], and mesentericin Y105 [19]), and *Pediococcus* (pediocin PA-1 [20]) and, more recently, in *Enterococcus faecium* (enterocins A [4], B [8], and P [10]). The bacteriocins produced by *Enterococcus* species (the enterocins) show considerable diversity. Based on their amino acid sequence similarity and their inhibitory spectra, most of these bacteriocins have been included in the pediocin-like group (36). According to the Klaenhammer classification, this group belongs to the class II bacteriocins, the small heat-stable nonlantibiotic bacteriocins (30). Although there are also lantibiotic enterocins that belong to the class I bacteriocins (7) and cyclic enterocins (45), they all have in common their pediocin-like broad spectrum of activity. Consequently, the enterocins have become attractive in recent years as natural additives for food preservation and safety (13).

All bacteriocins known to date are synthetized as prepeptides with an N-terminal leader sequence that directs their export outside the cell and that is removed before the active bacteriocin can be detected (36). Bacteriocins can be secreted by an ATP-binding cassette transport system (18) or by the general secretory pathway (10, 41, 49, 52). Bacteriocins that use the first system have a leader peptide that contains a conserved double-glycine motif that serves as a signal for processing and secretion (50). The leader peptide for the general secretory pathway is usually positively charged and has a hydrophobic core and a cleavage region (16, 23, 51). This peptide is processed by a signal peptidase during translocation across the cytoplasmic membrane (41, 51).

The organization of the genes for bacteriocin production and immunity is generally highly conserved, with the bacteriocin structural gene followed by a cotranscribed open reading frame (ORF) that encodes a putative immunity protein (30, 36). However, there are exceptions: the structural genes for carnobacteriocin A (52) and enterocin B (8) are followed by a putative *rho*-independent terminator with no recognizable ORF downstream.

In this paper, we describe the identification, purification, and genetic characterization of a new plasmid-carried enterocin, enterocin I (ENTI). ENTI has the same inhibitory spectrum as the pediocin-like enterocins but does not show significant sequence similarity with these bacteriocins. Other unusual features of ENTI are also described.

MATERIALS AND METHODS

Bacterial strains and media. The ENTI producer *E. faecium* 6T1a was isolated from a Spanish-style green olive fermentation. It was maintained as a frozen stock at −20°C in distilled water plus 20% (vol/vol) glycerol and propagated twice in MRS broth (Oxoid, Basingstoke, Hampshire, England) at 30°C before use.

The bacterial strains used as indicator organisms for the evaluation of ENTI

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TABLE 1. Inhibitory spectrum of ENTI from *E. faecium* 6T1a for gram-positive bacteria

Indicator species	Strain	Source[a]	Diam of inhibition (mm)
Bacillus cereus	9139	ATCC	8
B. subtilis	BD630	TNO	11.5
B. subtilis	1A510	BGSC	10
Clostridium sporogenes	C 22/10	TNO	12
Enterococcus faecalis	1353	NCDO	12
E. faecalis	BM 4100 WT	TNO	13
E. faecalis	610	NCDO	16
E. faecalis	19433	ATCC	16
Lactobacillus fermentum	9338	ATCC	11
L. fermentum	14933	ATCC	16
L. plantarum	128/2	Our strain collection	19
L. plantarum	1/43/1	Our strain collection	17
L. plantarum	LPS-5	Our strain collection	19
L. plantarum	LPS-10	Our strain collection	14
Lactococcus lactis	MG 1363	TNO	10
L. Lactis	MG 1614	TNO	14
L. Lactis	CNRZ150	INRA	20
L. Lactis	CNRZ117	INRA	9
L. Lactis	CNRZ148	INRA	15
Leuconostoc mesenteroides	808	NCDO	0
L. mesenteroides	CNRZ8015	INRA	0
Listeria innocua	BL86/26	TNO	9
L. monocytogenes	7973	NCTC	13
L. monocytogenes	L15sv 1/2	FVM	0
L. monocytogenes	5105	NCTC	14.3
L. monocytogenes	LI1sv 4	FVM	13
L. monocytogenes	Scott A	FVM	14.3
Pediococcus pentosaceus	FBB63	TNO	13
P. Pentosaceus	PC1	TNO	9
Propionibacterium acidi-propionici	563	NCDO	0
Propionibacterium spp.	P6	Our strain collection	9

[a] Abbreviations: ATCC, American Type Culture Collection (Rockville, Md.); BGSC, *Bacillus* Genetic Stock Center (Ohio State University, Columbus); FVM, Facultad de Veterinaria, Universidad Complutense (Madrid, Spain); INRA, Station de Recherches Laitières (Jouy-en-Josas, France); NCDO, National Collection of Dairy Organisms (Reading, United Kingdom); NCTC, National Collection of Type Cultures, Central Public Health Laboratory (London, United Kingdom); TNO, Nutrition and Food Research (Zeist, The Netherlands).

activity are listed in Table 1. All LAB used, as well as the *Enterococcus faecalis* strains, were propagated in MRS broth at 30°C. *Listeria* and *Bacillus* strains were grown in brain heart infusion (Oxoid) at 30 and 37°C, respectively. Clostridia were cultivated anaerobically in RCM medium (Difco Laboratories, Detroit, Mich.) at 37°C. *Propionibacterium* strains were propagated anaerobically in YGL medium (6) at 37°C. Gram-negative bacteria were propagated in tryptone soya broth (Oxoid) at 37°C. *Escherichia coli* DH5α (3) was used for all genetic manipulations.

Bacterial characterization. *E. faecium* 6T1a was examined by phase-contrast microscopy for cell morphology determination and by Gram staining. The strain was identified as *E. faecium* 6T1a by use of the Gram-Positive Identification Card (BioMérieux Vitek, Inc., Hazelwood, Mo.) in conjunction with the Vitek System (BioMérieux) for automated identification and by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) protein pattern analysis.

The Gram-Positive Identification Card included tests for growth on and resistance to bacitracin, optochin (ethylhydrocupreine hydrochloride), and novobiocin (ethylhydrocupreine hydrochloride), tolerance of bile (10 and 40%) and NaCl (6%); reduction of tetrazolium red; esculin hydrolysis; arginine hydrolase, catalase, urease, beta-hemolytic, and coagulase activities; and fermentation of dextrose, lactose, mannitol, raffinose, salicin, sorbitol, sucrose, trehalose, arabinose, hemicellulose, pyruvic acid, pullulan, inulin, melibiose, melezitose, cellobiose, ribose, and xylose.

Preparation of cell extracts and SDS-PAGE were carried out by the Research Group of the Laboratory for Microbiology (Ghent University) (39). The normalized and digitized protein patterns were numerically analyzed and clustered with the reference profiles in the LAB database (29, 40).

Bacteriocin assays. The bacteriocin producer 6T1a was grown in MRS broth at 30°C. The supernatant from late-log-phase cultures was adjusted to pH 7.0 with 5 M NaOH and filter sterilized through a 0.22-μm-pore-size Millex-GV filter (Millipore SA, Molsheim, France). The antimicrobial activity of the supernatant was determined by the well diffusion method (47). Fifty microliters of the supernatant was placed in wells (6 mm in diameter) cut in MRS, brain heart infusion, RCM, or YGL agar plates (25 ml) seeded (ca. 10^5 CFU/ml) with the indicator microorganisms listed in Table 1. The plates were kept for 2 to 4 h at 4°C to allow diffusion of the supernatants and then were incubated at 30 or 37°C for 18 h; the diameters of the zones of growth inhibition were then measured.

During purification, ENTI activity was quantified with a microtiter plate assay system (14). *E. faecium* 20, a non-enterocin-producing, enterocin-sensitive *E. faecium* 6T1a derivative obtained as described below, was used as the indicator strain. Each well of the microtiter plate contained 25 μl of twofold-concentrated MRS broth, 25 μl of ENTI fractions at serial two- or threefold dilutions, and 10 μl of the indicator strain (A_{600}, 0.01 [ca. 10^6 CFU/ml]). As a turbidity control, *E. faecium* 20 was incubated as described above but with sterile distilled water in place of the ENTI fractions. The microtiter plate cultures were incubated for 7 h at 37°C, after which growth inhibition of the indicator strain was measured spectrophotometrically at 600 nm with a microplate reader (model 450; Bio-Rad Laboratories, Hercules, Calif.). One ENTI unit (ENTIU) was arbitrarily defined as the amount of ENTI that inhibited the growth of the indicator strain by 50% (50% of the turbidity of the control culture without bacteriocin). This amount was expressed as the reciprocal of the highest dilution exhibiting 50% inhibition of the indicator strain per milliliter (ENTIU per milliliter). The results obtained with the two- and threefold dilution series for every sample were averaged. This method was also used to study the stability of the bacteriocin in the presence of heat and enzymes.

Sensitivity of ENTI to heat and enzymes. Cell-free, filter-sterilized, log-phase *E. faecium* 6T1a MRS culture supernatants were neutralized with 5 M NaOH and treated with solid ammonium sulfate (80% saturation at 0°C). The mixture was stirred for 2 h at 4°C and centrifuged at 20,000 × *g* for 30 min at 4°C. The precipitate was resuspended in citrate-phosphate buffer (50 mM, pH 5.0) and then desalted through PD10 gel filtration columns (Pharmacia Biotech, Uppsala, Sweden) equilibrated with the same buffer.

To test for heat sensitivity, samples containing 20,000 ENTIU/ml were heated to 100°C for 5 min or autoclaved (121°C, 1 atm) for 1, 5, 10, and 20 min, and the remaining activity was determined with the microtiter plate assay with *Lactobacillus plantarum* 128/2 as the indicator strain (25).

To test for enzyme sensitivity, samples containing 3,200 ENTIU/ml were treated with trypsin, pronase E, α-chymotrypsin, thermolysin, subtilopeptidase A, proteinase K, lysozyme, RNase A, α-amylase, papain, lipase, or ficin at a final concentration of 0.1 mg/ml. Buffers used were those recommended by the supplier (Sigma Chemical Co., St. Louis, Mo.). Samples were incubated at 37°C for 1 h, and the residual activity was determined with the microtiter plate assay. To exclude potential inhibition by hydrogen peroxide, a sample was treated with catalase (Sigma) at a final concentration of 100 U/ml (37). It was maintained at 25°C for 35 min, and its ENTI activity was then determined.

Bacteriocin purification. All the purification steps were carried out at room temperature, and all of the chromatographic equipment and media were purchased from Pharmacia Biotech. The bacteriocin was purified from a 1-liter MRS broth culture of *E. faecium* 6T1a by the same method as that described for the bacteriocin plantaricin S (26); fractions showing activity after the C_2/C_{18} reverse-phase column step were pooled and subjected to a second run. ENTI activity was eluted with 3 ml of 30% 2-propanol containing 0.1% trifluoroacetic acid, and the samples were stored at −80°C.

SDS-PAGE. C_2/C_{18} reverse-phase column-purified ENTI was analyzed by SDS-PAGE (46) with an 18.5% acrylamide resolving gel. A molecular weight marker (range, 2,512 to 16,946) kit (Pharmacia Biotech) was used for size standards. After electrophoresis, the gel was divided in two; one part was silver stained (34), and the other was used for the detection of antimicrobial activity (6) with *L. plantarum* 128/2 as the indicator strain.

N-terminal amino acid sequencing. Amino acid sequencing was performed by automated Edman degradation with a Beckman LF3000 sequencer/phenylthiohydantoin amino acid analyzer (System Gold) by F. Canals, Institut de Biologia Fonamental "Vicent Villar Palasí," University of Barcelona, Barcelona, Spain.

Plasmid profiles and curing of plasmids from *E. faecium* 6T1a. The protocol of Anderson and McKay (2) for isolating large-plasmid DNA from lactic streptococci was followed. *L. plantarum* LPCO10 was used as a source of plasmid markers (44).

To test if ENTI production and immunity were plasmid determined, novobiocin (0.125 to 0.5 μg/ml) and ethidium bromide (10 to 50 μg/ml) were used to treat MRS broth cultures of *E. faecium* 6T1a as described previously (44). Cultures were plated on MRS agar plates to yield individual colonies. After 18 h at 30°C, MRS soft agar (0.75% agar) containing the indicator strain *L. plantarum* 128/2 (final concentration, ca. 10^5 CFU/ml) was poured onto the plates, which were incubated for an additional 24 h. Colonies without clear zones of inhibition were purified on MRS agar and repeatedly transferred into MRS broth, and their ability to inhibit the growth of *L. plantarum* 128/2 was determined. As controls, MRS broth cultures of *E. faecium* 6T1a that had not been treated with novobiocin or ethidium bromide were processed at the same time. The immunity of the nonproducing variants to ENTI was examined by spotting active *E. faecium* 6T1a MRS culture supernatants on lawns of these derivatives. Plasmid DNA was isolated from both producing and nonproducing variants of *E. faecium* and analyzed.

Other DNA manipulations. Total DNA was prepared from *E. faecium* as described previously (9), and plasmid purification was done by CsCl gradient

TABLE 2. Purification of ENTI from *E. faecium* 6T1a

Fraction	Vol (ml)	Total A_{280}[a]	Total activity (10^7 BU)[b]	Sp act[c]	Increase in sp act	Yield (%)
Culture supernatant	1,000	33,273	0.22	67.3	1	100
Ammonium sulfate precipitation (fraction I)	67	2,640	1.10	4.2×10^3	62.4	500
Binding to SP-Sepharose fast flow (fraction II)	50	11.80	26.20	2.2×10^7	3.3×10^5	1.2×10^4
Binding to phenyl-Sepharose CL-4B (fraction III)	37	7.60	0.15	2.0×10^5	3.0×10^3	68.2
FPLC[d] (C_2/C_{18} reverse-phase chromatography)						
First run	12	2.60	0.20	7.7×10^5	1.1×10^4	91.0
Second run	3	1.84	2.10	1.1×10^7	1.7×10^5	954

[a] Total A_{280} is the A_{280} multiplied by the volume in milliliters.
[b] BU, bacteriocin units.
[c] Specific activity is BU divided by the A_{280}.
[d] FPLC, fast-performance liquid chromatography.

centrifugation. Isolation of *E. coli* plasmid DNA and subsequent nucleic acid manipulations were done as described by Maniatis et al. (32).

Cloning and sequencing of ENTI. From the amino acid sequence of ENTI, the degenerate primer ent1 (5′-GGNGAYCCNATHGTNAARAAR-3′) was designed and synthesized (Pharmacia Biotech). This oligonucleotide was 3′ end labelled with fluorescein-11-dUTP by use of a 3′-end-labelling ECL kit (Amersham Life Science, Little Chalfont, Buckinghamshire, United Kingdom) in accordance with the manufacturer's instructions and was used as a probe in Southern blot analysis of CsCl-purified, *Hin*dIII-digested plasmid pEF1. Hybridization and detection conditions were in accordance with the manufacturer's instructions. Nucleotide sequencing was performed by the MediGene Sequencing Service (Martinsried, Germany) with standard primers and primers designed from the deduced sequence (see Fig. 4). Analysis of ORFs and amino acid alignments were performed with programs in the Sequence Analysis Software Package (version 9.0) licensed from the Genetics Computer Group, University of Wisconsin, Madison (12).

RNA isolation and Northern blotting. Total RNA was isolated from *E. faecium* 6T1a cultures grown at 30°C in MRS broth at different phases of growth by the method of Anba et al. (1). Northern blot analysis was done as described by Ausubel et al. (3) with 40 μg of RNA from each sample. The RNA was blotted onto nylon membranes (Pharmacia Biotech), which were stained with methylene blue (0.02% [wt/vol] in sodium acetate [0.5 M, pH 5.2]) to check the amount of RNA transferred. RNA molecular weight marker I (Boehringer GmbH, Mannheim, Germany) was used to provide size standards. The 0.7-kb *Eco*RV-*Hin*dIII fragment contained in plasmid pSIG108 (see Fig. 4B) was used as a probe after being labelled with [α-^{32}P]CTP by use of a Ready-to-Go labelling kit (Pharmacia Biotech). Hybridization was carried out at 55°C for 16 h.

Nucleotide sequence accession number. The nucleotide sequence presented in this article has EMBL accession no. Y16413.

RESULTS

Identification of bacteriocin-producing strain 6T1a. The isolated bacteriocinogenic strain was a gram-positive, catalase-negative coccus which fermented glucose but did not produce gas. The strain was also able to ferment L-arabinose, cellobiose, mannitol, melibiose, ribose, sucrose, and trehalose but did not ferment inulin, lactose, melezitose, pullulan, D-raffinose, sorbitol, or xylose. Furthermore, it was able to grow in the presence of 6% NaCl; was resistant to bacitracin, bile, esculin, and optochin; produced ammonia from the hydrolysis of arginine; and could not reduce triphenyltetrazolium chloride. The strain did not show urease activity, and it was not betahemolytic. All of these characteristics, together with the SDS-PAGE protein pattern analysis (29, 39, 40), identified strain 6T1a as an *E. faecium* isolate.

Antimicrobial spectrum of ENTI. The antimicrobial activity of ENTI, determined by the agar diffusion assay, is summarized in Table 1. The *E. faecium* 6T1a inhibitory activity was directed against the natural flora present in olive fermentations, including *Lactobacillus* spp., *Lactococcus lactis*, and *Pediococcus pentosaceus* strains. Among gram-positive olive spoilage organisms, the compound of interest showed activity against *Propionibacterium* spp., *Clostridium* spp., and *Bacillus* strains. *E. faecalis*, a frequent contaminant in olive fermentations, was also sensitive to the bacteriocin. The bacteriocin also inhibited *Listeria innocula* and four of five *L. monocytogenes* strains tested. Gram-negative bacteria (*E. coli*, *Klebsiella* spp., and *Pseudomonas* spp.) were not inhibited by ENTI (data not shown).

Sensitivity of ENTI to heat and enzymes. ENTI was completely stable in the presence of heating at 100°C for 5 min but was partially inactivated by autoclaving. Thus, after 1 min of treatment, the activity was reduced from 20,000 to 5,000 ENTIU/ml, and prolonged autoclaving (5, 10, and 20 min) resulted in 2,500 ENTIU of residual activity per ml.

The inhibitory activity of ENTI was completely abolished after treatment with α-chymotrypsin, pronase E, proteinase K, subtilopeptidase A, thermolysin, and trypsin, thus suggesting a proteinaceous nature for the inhibitory compound. Other enzymes, such as α-amylase, catalase, ficin, lysozyme, and RNase A, did not affect the activity of ENTI.

Purification of ENTI. Maximum inhibitory activity in the growth medium was observed during the early stationary phase of growth (data not shown). The purification scheme for ENTI is shown in Table 2. After the second reverse-phase chromatographic step, a final yield of 954% the initial activity and a 170,000-fold increase in the specific activity of ENTI was obtained. The overall purification procedure resulted in a single peak upon C_2/C_{18} reverse-phase liquid chromatography (Fig. 1). SDS-PAGE analysis showed an electrophoretically pure protein with an apparent molecular size of ca. 5 kDa and with inhibitory activity against *L. plantarum* 128/2 (Fig. 2).

N-terminal sequencing of purified ENTI allowed determination of the first 40 amino acid residues (see Fig. 5), which revealed a highly hydrophobic protein, as expected.

Plasmid-curing experiments. After treatment with novobiocin or ethidium bromide, 2,637 or 683 colonies, respectively, of strain 6T1a were tested for ENTI production on MRS agar. Totals of 21.5 and 2.5% of the colonies from the novobiocin- and ethidium bromide-treated cultures, respectively, failed to induce clear zones of inhibition of lawns of *L. plantarum* 128/2. A total of 1,583 isolated colonies of 6T1a that had not been treated with a plasmid-curing agent were also tested for ENTI production; 15 of these (0.95%) lost inhibitory activity. When the suspected non-ENTI producers were purified on MRS agar and repeatedly subcultured in MRS broth, none of them regained the ability to produce ENTI. All of these ENTI-deficient derivatives were also sensitive to ENTI.

Plasmid profile analysis of the non-ENTI-producing derivatives isolated after novobiocin or ethidium bromide treatment, as well as those appearing spontaneously, showed that the 23-kb plasmid pEF1 harbored by parental strain 6T1a had been lost in all cases (Fig. 3).

Genetic analysis and DNA sequencing of *entI*. Southern analysis of restriction fragments of pEF1 with the degenerate oligonucleotide ent1 confirmed that the *entI* structural gene was located on pEF1 (data not shown). A 2.5-kb *Hin*dIII re-

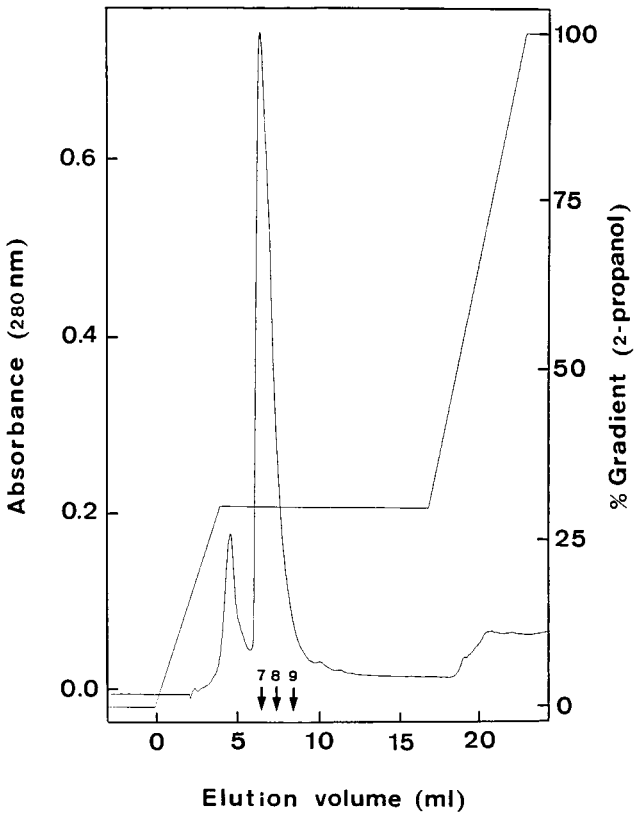

FIG. 1. C_2/C_{18} reverse-phase chromatography of ENTI (fraction V). Numbers above the arrows indicate the fractions (1 ml each) exhibiting ENTI activity. Maximum bacteriocin activity was detected in fraction 8.

striction fragment from pEF1 that hybridized to ent1 was identified, purified from an agarose gel, and ligated with *Hin*dIII-cleaved pUC18 to give the recombinant plasmid pSIG106 (Fig. 4). By use of an internal *Eco*RV restriction site in the 2.5-kb *Hin*dIII fragment, 1.8-kb *Hin*dIII-*Eco*RV and 0.7-kb *Eco*RV-*Hin*dIII fragments were cloned separately into pBluescript II SK(+), giving the recombinant plasmids pSIG107 and pSIG108, respectively (Fig. 4).

Analysis of the sequences of the inserts revealed several ORFs preceded by putative ribosome binding sites (Fig. 5). Two direct repeats of 15 nucleotides separated by 1 nucleotide, with the consensus sequence 5′-AAATATxTxTTTTGT-3′, are present 55 nucleotides upstream of the first ORF, named *entI*, which codes for a protein identical to ENTI. The protein encoded by *entI* has a molecular weight of 5,190 and a theoretical pI of 10.82. The N-terminal sequence of the protein deduced from the nucleotide sequence revealed the absence of any kind of leader peptide. A second ORF, ORF2, which encodes a putative protein that is very similar to ENTI (72.7% identity), is located 19 bp downstream of *entI*. ORF2 encodes a 5,178-Da protein with a theoretical pI of 11.0. An inverted repeat which may function as a rho-independent transcription terminator was found 27 nucleotides downstream of ORF2.

A 1,254-bp insertion sequence (IS)-like element lies downstream of ORF2 (Fig. 5). Two 41-bp imperfect inverted repeats flank a DNA sequence containing an ORF (ORF3). ORF3 encodes a protein of 366 amino acids that shows 36% identity to the transposase of the lactococcal insertion element IS*904* (43). Identities of 30 to 36% were found with other known transposases (reference 31 and data not shown). The number of copies of the IS-like element in the genome of *E. faecium*

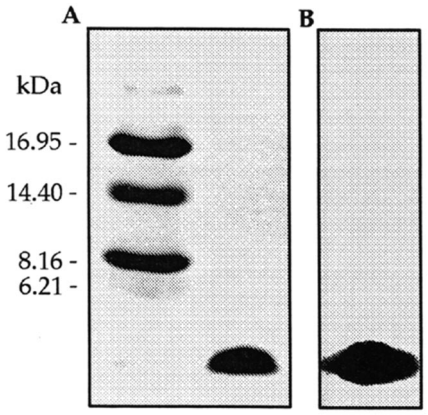

FIG. 2. SDS-PAGE of ENTI and detection of antimicrobial activity. (A) Silver-stained gel. Left lane, size standards (sizes indicated on the left); right lane, purified ENTI sample. (B) Gel fixed in 20% isopropanol–10% acetic acid and washed in deionized water as described by Bhunia et al. (6). The gel was then placed on an MRS agar plate and overlaid with MRS soft agar containing *L. plantarum* 128/2.

6T1a was then determined. Total DNA from strain 6T1a was digested with several restriction enzymes, and the resulting fragments were analyzed by Southern hybridization with oligonucleotide ent7 as a probe (Fig. 5). The results revealed that only one copy of the putative IS-like element was present in 6T1a and that it was located on pEF1 (data not shown). Finally, an incomplete ORF (ORF5) was identified downstream of the IS-like element; this ORF is transcribed in the direction opposite that of the putative transposase. The putative protein encoded by ORF5 is homologous to proteins involved in the control of plasmid partitioning (protein STBA) (15).

Transcription analysis of the sequenced ORFs. Northern analysis performed on total RNA isolated from *E. faecium* 6T1a at different times of culturing in MRS broth at 30°C showed a unique mRNA of ca. 0.35 kb (Fig. 6). This transcript is long enough to encode both *entI* and ORF2. The level of this

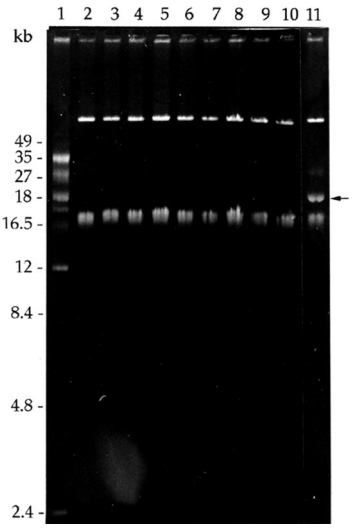

FIG. 3. Agarose gel electrophoresis of plasmid DNAs from novobiocin- and ethidium bromide-treated variants of *E. faecium* 6T1a. Lane 1, plasmid profile from *L. plantarum* LPCO10 used as a size marker (sizes indicated on the left); lanes 2 to 10, non-ENTI-producing, ENTI-sensitive variants from *E. faecium* 6T1a lacking the 23-kb plasmid pEF1; lane 11, *E. faecium* 6T1a (the pEF1 plasmid is indicated by an arrow).

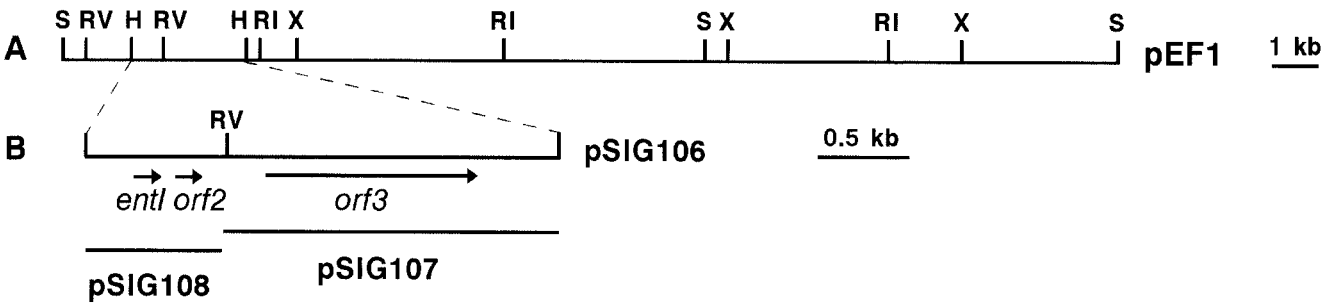

FIG. 4. Restriction map of pEF1 (A) and pSIG subclones (B) containing the *entI* locus. Restriction sites: H, *Hin*dIII; RI, *Eco*RI; RV, *Eco*RV; S, *Sal*I; X, *Xba*I.

mRNA reached a maximum at the end of logarithmic growth (32,000 ENTIU/ml) and then declined during the stationary phase, although the level of ENTI activity remained constant (16,000 ENTIU/ml).

DISCUSSION

In this paper, we have described the purification and genetic characterization of a new plasmid-carried bacteriocin produced by *E. faecium* 6T1a, which was isolated from a Spanish-style green olive fermentation. To our knowledge, this is the first time that a bacteriocinogenic member of the genus *Enterococcus* has been isolated from such a fermentation. Almost nothing is known about the role of this genus in fermentation, but the spectrum of inhibitory activity of ENTI suggests a potentially useful means for controlling the growth of spoilage microorganisms that are often found in olive brines.

ENTI exhibits a broad inhibitory spectrum that includes most of the gram-positive bacteria that constitute the natural flora present in olive fermentations. Like other enterococcal bacteriocins and other members of the pediocin-like family of bacteriocins (4, 7, 8, 10, 30, 36, 45), ENTI strongly inhibited several *L. monocytogenes* strains. In addition, ENTI also inhibited several *L. monocytogenes* strains tested, including the nisin-producing strains CNRZ148 and CNRZ150, whereas other enterococcal bacteriocins are not active against lactococci.

Purification of ENTI was accomplished with the protocol described for plantaricin S (26), consistent with the conserved biochemical characteristics of many bacteriocins; e.g., they are generally small, cationic, and highly hydrophobic. As has been noted for other bacteriocins (4, 5, 10, 26, 27, 35, 38), a marked increase in specific activity occurred after some of the purification steps. This finding presumably reflects the presence in culture supernatants of inhibitory compounds that are removed during ENTI purification and/or the dissociation of high-molecular-weight ENTI aggregates into their smaller, more active forms.

Amino acid sequence comparisons indicated that ENTI is very different from other enterococcal and pediocin-like bacteriocins (4, 7, 8, 10, 17, 19, 20, 21, 42, 45, 48, 52). Thus, ENTI does not contain the highly conserved YGNGVxC motif found in the N-terminal part of most of these bacteriocins (enterocin B [8] and carnobacteriocin A [52] are also exceptions). ENTI also lacks cysteine residues, which are always present in the pediocin-like bacteriocins, including the enterocins. Cys content has been related to the antibacterial efficiency of bacteriocins (24); those with two or more cysteines capable of forming disulfide bridges have a wide inhibitory spectrum, while those with no cysteines have a narrow inhibitory spectrum. Within the enterocins, enterocin 4, bacteriocin AS-48 (28, 33, 45), and ENTI are the only bacteriocins that do not contain cysteine but show a broad inhibitory spectrum. Together with the absence of significant amino acid sequence similarity to other bacteriocins, this finding suggests that the mechanism of action of ENTI may be different from that proposed for the pediocin-like family.

Cloning and sequencing of the structural gene *entI* revealed some unusual features about the enterocin and its genetic organization. Whereas all bacteriocins described thus far have a leader peptide, the N terminus of ENTI deduced from the nucleotide sequence was identical to that obtained from amino acid sequencing, indicating the absence of a leader sequence. Such leader peptides are believed to be signals for export and processing, through the use of either an ATB-binding cassette-type or a *sec*-dependent transport system (30, 36). Thus, the mechanism by which ENTI is transported outside the cell is unknown and is likely to be novel.

Production of and immunity to ENTI are plasmid associated, as the loss of plasmid pEF1 led to phenotypes of non-ENTI production and ENTI sensitivity. Unusual also was that no recognizable immunity gene was found downstream of *entI*; instead, ORF2, which encodes an ENTI homolog, was found. Perhaps the protein encoded by ORF2 functions as an immunity protein. Based on its homology to ENTI, this protein may bind to the putative receptors for ENTI which would be present on the surface of 6T1a cells, thus preventing ENTI binding and providing immunity to ENTI. Further experiments are in progress to identify the protein(s) that confers immunity to ENTI.

The nucleotide sequence downstream of the *entI* locus appeared to contain an IS-like element. Many IS elements have been described for LAB, with most strains carrying multiple copies of at least two (11). It is interesting to note that nisin production is associated with a 68-kb transposon that is flanked by two IS*904* elements (43). In *E. faecium* 6T1a, the *entI* locus does not seem to be associated with a transposon, since the IS-like sequence was present only once in pEF1. Whether the IS-like element is active or not is unknown. The absence of direct repeats flanking the IS-like element suggested that these flanking regions may have undergone secondary mutations following insertion.

The work presented here increases our knowledge about the bacteriocins made by LAB and suggests that ENTI represents a novel class of bacteriocins. This conclusion is based on the absence of any signal peptide, which could indicate that its secretion mechanism is different from any other previously described secretion systems for the known bacteriocins. This fact may prove advantageous for the heterologous expression of ENTI in other bacteria. Another attractive feature of ENTI is its plasmid-carried nature. Thus, pEF1 could be used as a cloning vector in *E. faecium* to produce not only ENTI but also

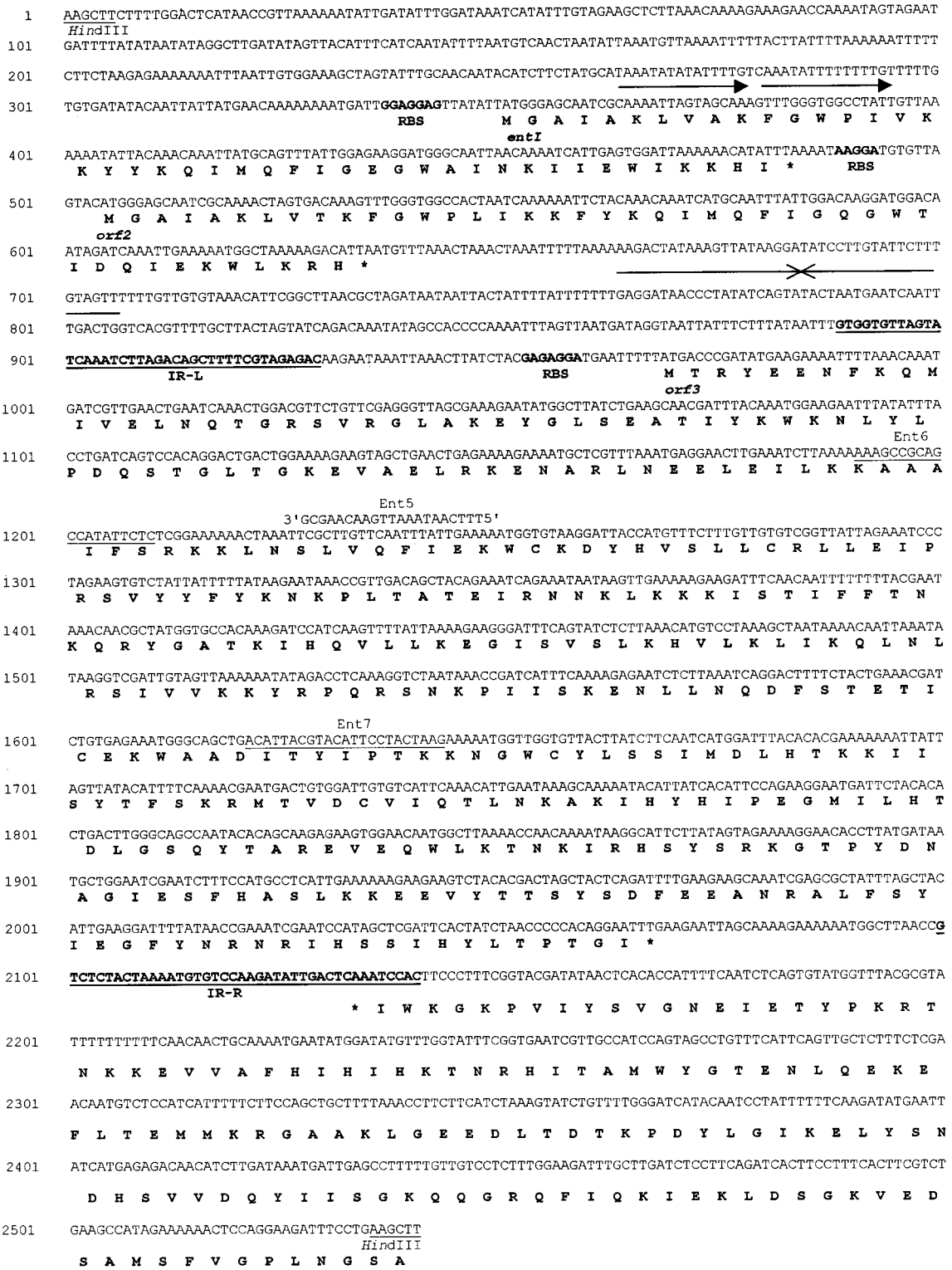

FIG. 5. Nucleotide sequence of the *entI* locus and deduced protein sequences. Ribosome binding sites (RBS) are indicated in boldface letters. Direct repeats upstream of *entI* are indicated by arrows. Sequences that have dyad symmetry and might serve as transcription terminators are indicated by facing arrows downstream of orf2. Inverted repeats upstream and downstream of ORF3 that could serve as the right (IR-R) and left (IR-L) arms for an IS-like element are underlined and shown in boldface letters. Oligonucleotides used for sequencing are shown. Oligonucleotides Ent6 and Ent7 used for sequencing are underlined. Other oligonucleotides (e.g., Ent5) are shown.

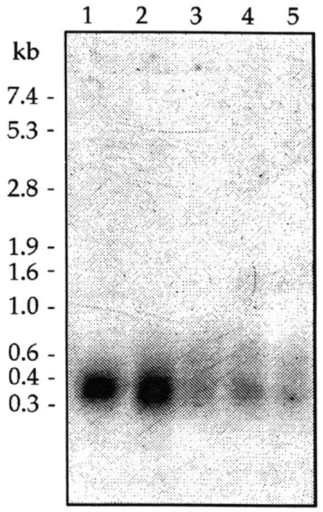

FIG. 6. Northern blot analysis of the *entI* operon. The purified DNA fragment that was cloned in pSIG108 and that contained both *entI* and *orf2* was used as a probe. RNA molecular weight markers are indicated on the left. Forty micrograms of RNA was loaded in each lane. Lane 1, log phase of growth; lane 2, transition from log to stationary phase; lane 3, early stationary phase; lanes 4 and 5, late stationary phase (25 and 40 h of incubation, respectively).

other broad-spectrum bacteriocins to overcome bacterial resistance to many bacteriocins. With these facts and ideas this in mind, studies to define the minimal replicon of pEF1 in *E. faecium* 6T1a are in progress. Finally, the IS-like element found in pEF1 could be a potential tool for mutagenesis in *E. faecium* 6T1a.

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ADDENDUM

While the manuscript was under review, a paper dealing with the same topic by Cintas et al. was published (10a); our results are basically in agreement, except for ORF2, which was described by them as encoding enterocin L50B.

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