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Bacteriocin T8, a Novel Class IIa *sec*-Dependent Bacteriocin Produced by *Enterococcus faecium* T8, Isolated from Vaginal Secretions of Children Infected with Human Immunodeficiency Virus

M. De Kwaadsteniet, T. Fraser, C. A. Van Reenen, and L. M. T. Dicks*

Department of Microbiology, Stellenbosch University, Stellenbosch, South Africa

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***Enterococcus faecium* T8, isolated from vaginal secretions of children with human immunodeficiency virus, produces a class IIa *sec*-dependent bacteriocin that is structurally different from three other class IIa *sec*-dependent bacteriocins, i.e., enterocin P and an enterocin P-like bacteriocin, produced by *Enterococcus faecium*, and bacteriocin 31, produced by *Enterococcus faecalis*, and from a class III bacteriocin produced by *E. faecalis*. The genes encoding the bacteriocin, immunity protein, mobilization protein, and relaxase nuclease are located on a 7-kb plasmid. Bacteriocin T8 has a molecular mass of 5.1 kDa based on its DNA sequence, similar to the 5.0 kDa recorded for bacteriocin 31 but larger than the 4.6 kDa reported for enterocin P. At the amino acid level, bacteriocin T8 is 69% homologous to bacteriocin 31 and 47% homologous to enterocin P. Bacteriocin T8 is active against *E. faecalis* isolated from patients diagnosed with vaginosis, against *Lactobacillus sakei*, and against a *Propionibacterium* sp. The peptide is heat stable (60 min at 100°C) and remains active in phosphate buffer from pH 4.0 to 10.0. The mode of activity is bactericidal, as determined with *E. faecalis*.**

Bacteriocins are ribosomally transcribed peptides that are antimicrobial toward closely related bacteria (13). Four classes of bacteriocins have been described based on their structural, chemical, and functional properties (13). Peptides from classes I and II are heat stable. Small, membrane-active, posttranslationally modified peptides containing lanthionine and β-methyl lanthionine are classified as lantibiotics and grouped into class I (13). Class II bacteriocins differ from class I in that they do not undergo posttranslational modification and have a $YGNGX_2C$ X_4CXV sequence at the N terminus (13). Single peptides (one-peptide bacteriocins) are grouped into class IIa. Typical examples of these are pediocin PA-1, produced by *Pediococcus acidilactici* (16); sakacin P, produced by *Lactobacillus sakei* (27); plantaricin 423, produced by *Lactobacillus plantarum* 423 (29); and enterocin A, produced by *Enterococcus faecium* (3). Two-peptide bacteriocins are grouped into class IIb. Large heat-labile bacteriocins are grouped into class III, and complex bacteriocins are grouped into class IV (13).

The majority of class IIa bacteriocins have a double-glycine sequence at the N terminus, which serves as a recognition signal for peptide procession and secretion (3, 16, 27, 29). ATP-binding cassette (ABC) transporters translocate the bacteriocin across the cell membrane (9). A few class IIa bacteriocins make use of a signal peptide instead of a double-glycine leader sequence (5, 28). The leader peptide is usually positively charged and has a hydrophobic core and cleavage region. The peptide is processed by a signal peptidase during translocation across the cell membrane (30). Enterocin P (5) and bacteriocin 31 (28), produced by *E. faecium* P13 and *Enterococcus faecalis* Y1717, respectively, are examples of *sec*-dependent class IIa bacteriocins. Other examples of *sec*-dependent peptides are the large heat-labile class III peptide enterolysin A, produced by *E. faecalis* LMG 2333 (20), and lactococcin 972, a two-peptide bacteriocin classified as class IIb (15). Two nonlantibiotics, divergicin A (31) and acidocin B (14), are also secreted by a signal peptide.

The purpose of this study was to characterize the bacteriocin produced by *E. faecium* T8 and compare it with other bacteriocins of the same class.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Lactic acid bacteria were propagated in De Man Rogosa and Sharpe (MRS) broth (Biolab Diagnostics, Midrand, South Africa) at 37°C. Uropathogenic strains of *E. faecalis* were cultured in brain heart infusion (BHI) broth (Biolab Diagnostics) at 37°C. Uropathogenic *E. faecalis* strains, including *E. faecalis* MDK2, were isolated from patients diagnosed with bacterial vaginosis. *Escherichia coli* Bluescript and DH5α were cultured in Luria-Bertani (LB) broth (Biolab Diagnostics) on a rotating wheel at 37°C. Other strains included in the test panel were obtained from Laboratorium voor Microbiologie (LMG), University of Ghent, Ghent, Belgium, and our own culture collection. *E. faecalis* BFE1071 was isolated from the feces of minipigs, *Lactobacillus salivarius* 241 was isolated from the ileum of a piglet, *Lactobacillus curvatus* DF38 was isolated from salami, and *Lactobacillus casei* LHS_3 was isolated from fortified wine. Bacteria were stored at −80°C in 40% (vol/vol) glycerol.

Identification of strain T8. Strain T8 was isolated from children infected with human immunodeficiency virus. Strain T8 was identified to the genus level by its phenotypic characteristics (10) and to the species level by 16S rRNA gene sequencing. DNA was isolated using a High Pure PCR preparation kit from Roche Diagnostics (Indianapolis, Ind.) and amplified by PCR using the 16S rRNA gene primers and conditions described by Felske et al. (8). Amplified fragments were cloned into the pGEM-T Easy vector system (Promega, Madison, Wis.). Constructs were transformed into *E. coli* DH5α. Plasmid DNAs were isolated using a QIAprep Spin miniprep kit (QIAGEN, Valencia, Calif.). DNAs were sequenced at the Central Analytical Facility, Stellenbosch University, on an automatic sequencer (ABI Genetic Analyzer 3130Xl; Applied Biosystems SA), using BigDye Terminator chemistry (Biosystems, Warrington, England). All ligation reactions and transformations were performed according to standard procedures (2). Homology with sequences in GenBank was determined by using the BLAST program (1).

* Corresponding author. Mailing address: Department of Microbiology, Stellenbosch University, Private Bag X1, Matieland 7602, Stellenbosch, South Africa. Phone: 27-21-808 5849. Fax: 27-21-808 5846. E-mail: lmtd@sun.ac.za.

TABLE 1. Primers used in this study

Primer	Sequence
M13F	GTTTTCCCAGTCACGAC
M13R	CAGGAAACAGCTATGAC
MDK1(a)	GTGTGTATTGCACCACTTAG
MDK1(b)	GAGGTTCAAGACAACTATGAG
MDK2(a)	GGATAGGCGACAAGTTATTA
MDK2(b)	TCGGTGAAATTGTTGCAAAT

Antimicrobial spectrum of bacteriocin T8. *Enterococcus faecium* T8 was cultured in 500 ml MRS broth (Biolab) for 24 h at 37°C. Cells were harvested (10,000 × *g*, 10 min, 4°C), and proteins were precipitated from the cell-free supernatant with 80% ammonium sulfate (22). The precipitate was collected by centrifugation (10,000 × *g*, 1 h, 4°C), and the pellet was resuspended in 10 ml ammonium acetate buffer (pH 6.5). The concentrated bacteriocin was dialyzed against 4 liters sterile distilled water, using a Spectra-Por membrane with a 1,000-Da cutoff (Spectrum Inc., CA). Antimicrobial activity was determined by using the agar spot test method (25) and the well diffusion method (26). The activity of the crude extract was expressed in arbitrary units per ml (AU/ml). One AU is defined as the reciprocal of the highest serial twofold dilution showing a clear zone of inhibition of the indicator strain (29). The crude extract was used to determine the spectrum of antimicrobial activity and to characterize bacteriocin T8.

Characterization of bacteriocin T8. One milliliter of crude extract containing bacteriocin T8 was incubated for 2 h at 30°C in the presence of 0.5 mg ml^{-1} (final concentration) amylase (Sigma Diagnostics, St. Louis, Mo.), proteinase K (Roche Diagnostics Corporation, Mannheim, Germany), pronase (Boehringer Mannheim GmbH, Germany), or pepsin (Boehringer Mannheim GmbH). The enzymes were inactivated (10 min at 80°C). A bacteriocin T8 crude extract not treated with enzymes was used as a control (11).

In a separate experiment, the crude extract of bacteriocin T8 was adjusted to pH values ranging from 2.0 to 12.0 (increments of 2 pH units) with sterile 1 M NaOH or 1 M HCl and incubated for 2 h at 37°C. The pH was then neutralized by slowly adding 1 M NaOH or 1 M HCl (11).

The cell-free supernatant of *E. faecium* T8 was incubated at 60°C or 100°C for 10, 30, and 90 min. The control was a cell-free supernatant of *E. faecium* T8 not subjected to heat treatment and kept at 8°C (11). All treated supernatants were tested for antimicrobial activity as described previously.

E. faecalis MDK2, isolated from patients diagnosed with vaginosis, was used as a sensitive strain in all bacteriocin activity tests.

The molecular size of bacteriocin T8 was determined by Tricine-sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (24). One half of the gel was stained with Coomassie blue R250 (Saarchem, Krugersdorp, South Africa). The other half was prewashed with sterile distilled water and overlaid with *E. faecalis* MDK2 (10^6 CFU ml^{-1}) to determine the position of bacteriocin T8 (29). A Rainbow protein molecular mass marker with fragments of 2.5 to 45.0 kDa (Amersham International, United Kingdom) was used.

Mode-of-action studies were performed to determine if the bacteriocin is bactericidal. Actively growing cells of *E. faecalis* MDK2 were inoculated (0.2% [vol/vol]) into BHI broth and incubated for 5 h at 37°C to mid-exponential growth phase. Bacteriocin T8 crude extract (6,400 AU ml^{-1}) was added to the culture, and cell density readings were recorded hourly at 600 nm for 8 h (11). The control was autoclaved (15 min at 121°C) bacteriocin T8.

Plasmid curing. Actively growing cells of *E. faecium* T8 were inoculated into MRS broth and supplemented with acridine orange (Merck), novobiocin (Sigma), or SDS (Sigma) (21). The components were added at 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0% (wt/vol). After 18 h of incubation at 37°C, cells from each treatment were plated onto MRS agar and incubated for a further 18 h. Colonies were replica plated onto MRS agar, and one set was overlaid with *E. faecalis* MDK2 embedded in 1% (wt/vol) soft agar (10^6 CFU ml^{-1}). The plates were incubated for 18 h at 37°C. Colonies with no inhibition zones were identified. Corresponding colonies were selected from replica plates, inoculated into MRS broth, and incubated for 18 h at 37°C. The cells were harvested (10,000 × *g*, 10 min, 4°C),

TABLE 2. Spectrum of antimicrobial activity of bacteriocin T8

Microorganism	Strain[a]	Growth conditions (temp [°C], oxygen requirement)	Growth medium	Bacteriocin activity
Enterococcus faecalis	MDK1	37, aerobic	BHI	+
	MDK2	37, aerobic	BHI	+
	MDK3	37, aerobic	BHI	+
	MDK4	37, aerobic	BHI	+
	MDK5	37, aerobic	BHI	+
Lactobacillus acidophilus	LMG 13550	37, anaerobic	MRS	−
Lactobacillus bulgaricus	LMG 13551	37, anaerobic	MRS	−
Lactobacillus casei	LMG 13552	37, anaerobic	MRS	−
	LHS_3	37, aerobic	MRS	−
Lactobacillus curvatus	LMG 13553	30, anaerobic	MRS	−
	DF38	37, aerobic	MRS	−
Lactobacillus fermentum	LMG 13554	37, anaerobic	MRS	−
Lactobacillus helveticus	LMG 13555	42, anaerobic	MRS	−
Lactobacillus plantarum	LMG 13556	37, anaerobic	MRS	−
Lactobacillus reuteri	LMG 13557	37, anaerobic	MRS	−
Lactobacillus sakei	LMG 13558	30, anaerobic	MRS	+
Lactobacillus salivarius	241	37, aerobic	MRS	−
Pediococcus pentosaceus	LMG 13560	30, anaerobic	MRS	−
	LMG 13561	30, anaerobic	MRS	−
Leuconostoc cremoris	LMG 13562	30, anaerobic	MRS	−
	LMG 13563	30, anaerobic	MRS	−
Streptococcus thermophilus	LMG 13564	42, anaerobic	MRS	−
	LMG 13565	42, anaerobic	MRS	−
Enterococcus faecalis	LMG 13566	37, aerobic	BHI	+
	BFE 1071	37, aerobic	MRS	+
Staphylococcus carnosus	LMG 13567	37, aerobic	BHI	−
Listeria innocua LMG	LMG 13568	30, aerobic	BHI	−
Bacillus cereus	LMG 13569	37, aerobic	BHI	−
Clostridium sporogenes	LMG 13570	37, anaerobic	RCM	−
Clostridium tyrobutyricum	LMG 13571	30, anaerobic	RCM	−
Propionibacterium sp.	LMG 13574	32, anaerobic	GYP	+

[a] BFE, Bundesforschungsanstalt für Ernährung, Karlsruhe, Germany.

TABLE 3. Effects of enzymes, pH, and temperature on the activity of bacteriocin T8

Treatment	Bacteriocin activity[a]
Enzymes (0.5 mg/ml)	
Amylase	+
Pepsin, pronase, or proteinase K	−
Control (not treated with enzymes, 30°C)	+
pH	
2	−
4, 6, 8, or 10	+
12	−
Control (pH not adjusted, 30°C)	+
Heat	
10, 30, or 90 min at 30°C	+
10, 30, or 90 min at 60°C	+
10 or 30 min at 100°C	+
90 min at 100°C	−
Control (bacteriocin at 4°C)	+

[a] +, activity; −, no activity.

and plasmid DNAs were isolated using a QIAGEN Plasmid Midi kit. Bacteriocin-producing cells of *E. faecium* T8 were used as a control.

Sequencing of plasmid T8. Plasmid DNA isolated from *E. faecium* T8 was digested with HindIII (Roche) and ligated with T4 ligase (Roche) into pBluescript SK(+/−) (Invitrogen, Paisley, United Kingdom). The construct was transformed into *E. coli* DH5α according to the method of Ausubel et al. (2). DNA was isolated from the transformants by using a QIAprep Spin miniprep kit (QIAGEN). After sequencing of the pBluescript construct, primers were designed to sequence the rest of the plasmid. Primers designed from sequenced fragments (Table 1, MDK1a and MDK1b) were used to amplify the DNA, which was then cloned into the pGEM-T Easy vector system (Promega). The constructs were transformed into *E. coli* DH5α. The DNA was isolated as described previously. Subsequent primers (Table 1, MDK2a and MDK2b) were designed from amplified DNA fragments, and the plasmid was sequenced by primer walking.

Nucleotide sequence accession number. The GenBank accession number for the sequences of the structural gene encoding bacteriocin T8 (ORF 1) and the potential immunity gene (ORF 2) is DQ402539.

RESULTS

Antimicrobial activity spectrum of bacteriocin T8. Bacteriocin T8 inhibited uropathogenic strains of *E. faecalis*, a *Lactobacillus* sp. strain, two *Enterococcus* sp. strains, and a *Propionibacterium* sp. strain (Table 2). The antimicrobial activity in the crude supernatant was 400 AU/ml and increased to 1,600 and 3,200 AU/ml after protein purification and concentration, respectively.

Identification of *E. faecium* T8. *E. faecium* T8 was morphologically related to *Enterococcus* sp. and revealed 94% 16S rRNA gene homology with *E. faecium* strain SF3 (gi 55442388 gb AY735408.1). Fermentation of glycerol and melibiose confirmed the classification of strain T8 as *E. faecium* (10).

Characterization of bacteriocin T8 and mode-of-action studies. Bacteriocin T8 was sensitive to proteinase K, pepsin, and pronase (Table 3). Treatment with amylase had no effect on bacteriocin activity. No activity was recorded at pHs 2 and 12 and after 90 min at 100°C. The addition of bacteriocin T8 to actively growing cells of *E. faecalis* resulted in a rapid decrease in cell density (Fig. 1). According to Tricine-SDS-polyacrylamide gel electrophoresis, the molecular size of bacteriocin T8 is between 3.5 and 6.5 kDa (not shown).

Plasmid curing and sequencing. No loss in bacteriocin activity was recorded when *E. faecium* T8 was incubated in the

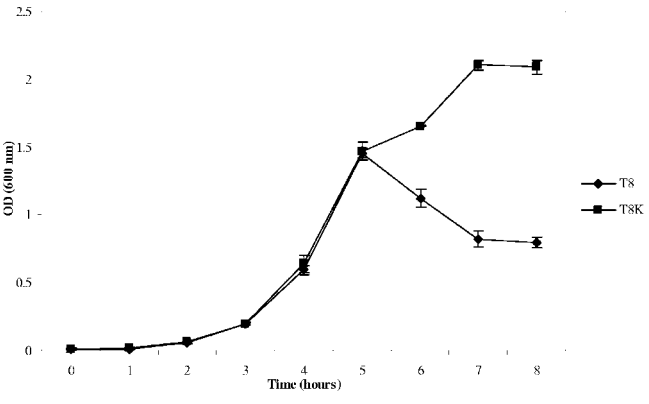

FIG. 1. Effect of bacteriocin T8 on cell growth of *E. faecalis* MDK2. Bacteriocin T8 was added to mid-logarithmic-phase cells (after 5 h of growth). Symbols: ■, growth in the absence of bacteriocin T8; ◆, growth in the presence of bacteriocin T8. OD, optical density.

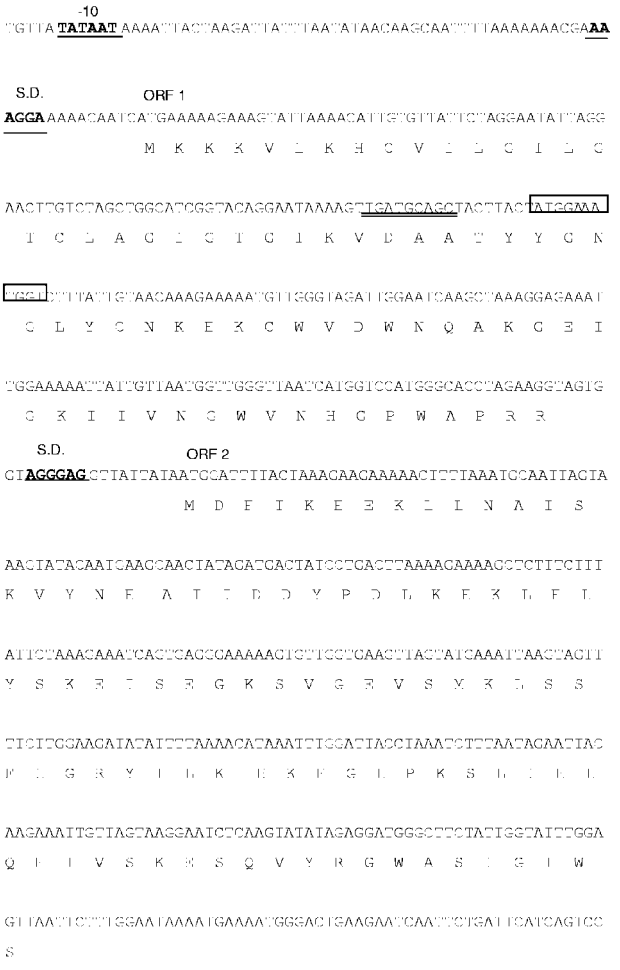

FIG. 2. Nucleotide sequence of the bacteriocin region and deduced amino acid sequence. The −10 promoter box and the Shine-Dalgarno (S.D.) ribosome-binding sequences are underlined. ORF 1 encodes the structural gene of bacteriocin T8, and ORF 2 encodes the potential immunity gene.

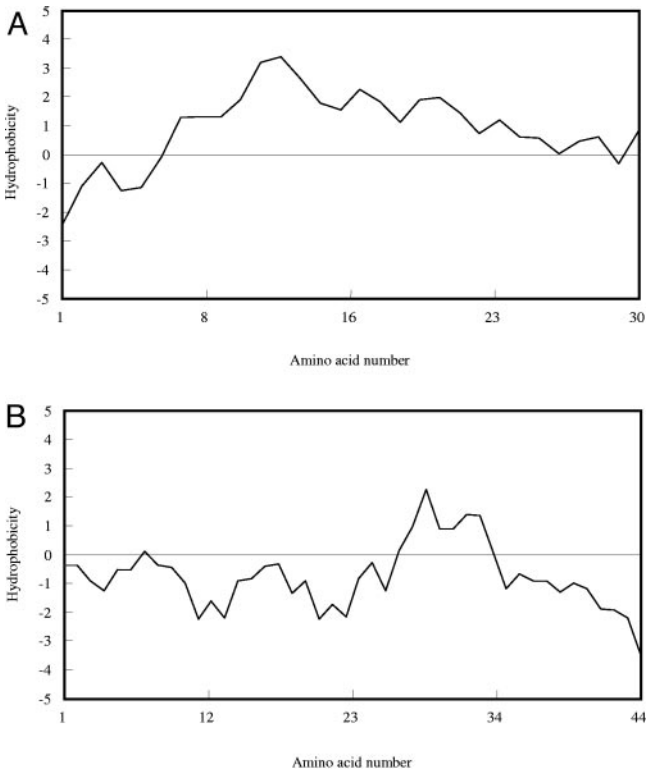

FIG. 3. Hydrophobicity profiles of the leader peptide (A) and the mature bacteriocin (B).

presence of acridine orange or novobiocin. However, growth was impaired in the presence of 3% (wt/vol) SDS, accompanied by a complete loss of bacteriocin T8 activity.

Two plasmids were isolated from *E. faecium* T8. Growth in the presence of 3% (wt/vol) SDS resulted in the loss of the smaller, 7-kb plasmid (plasmid T8, not shown) and a loss of bacteriocin activity. Sequencing of plasmid T8 revealed four open reading frames (ORFs). ORF 1 encodes a 74-amino-acid peptide, the bacteriocin precursor. As shown in Fig. 2, the start codon (ATG) is preceded 9 bp upstream by a potential Shine-Dalgarno ribosome-binding site (AAAGGA; underlined) and 68 bp upstream by a potential −10 consensus promoter region (Pribnow box) (TATAAT; underlined). The first 222 bp in ORF 1 encode the prebacteriocin (leader peptide and probacteriocin). The leader peptide contains a potential signal peptidase-processing site (VDA) from positions 82 to 90 (Fig. 2, double underlining). A conserved YGNG sequence is located at positions 100 to 111 (Fig. 2, box). Two cysteine residues (positions 118 to 120 and 133 to 135) and a valine residue (position 139 to 141) are also conserved (Fig. 2). The center of the signal peptide is hydrophobic (Fig. 3A), conforming to that of typical signal peptides (30). The mature peptide is hydrophilic overall, with minor hydrophobic peaks (Fig. 3B).

The second ORF (ORF 2) is preceded 10 bp upstream by a potential Shine-Dalgarno ribosome-binding site (AGGGAG; underlined in Fig. 2). ORF 2 encodes a 95-amino-acid peptide which resembles an immunity protein. ORF 3 encodes a mobilization protein that has 96% homology to the mobilization protein of *E. faecium* DO (gi 68194353 gb EAN08864.1). ORF 4 encodes a relaxase mobilization nuclease that has 75% homology with *E. faecium* DO (gi 68194349 gb EAN08860.1). ORF 4 is located immediately downstream of the mobilization gene.

DISCUSSION

Bacteriocin T8 is inactivated by proteolytic enzymes but not by amylase. This suggests either that the peptide is not glycosylated or that its activity is not influenced by glycosylation. According to its size (average, 5.07 kDa) and stability in heat (60 min at 100°C), bacteriocin T8 belongs to the class IIa group of bacteriocins.

Bacteriocin T8 differs from the majority of class IIa bacteriocins in having a *sec*-dependent leader signal peptide (Fig. 2 and 4). As far as we could determine, this is the fourth class IIa *sec*-dependent bacteriocin described and the fifth *sec*-dependent bacteriocin described for a species of the genus *Enterococcus*. The three class IIa *sec*-dependent bacteriocins de-

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                    i          ii          iii
Enterocin P         MRKKLFSLALIGI---FGLVVTNFGTKVDA              27
Bacteriocin 31      MKKKVICGIIGIG-------FTALGTNVEA              24
Bacteriocin T8      MKKKVLKHCVILGILGICLAGIGTGIKVDA              30
(A)

Enterocin P       ATRSYGNGVYCNNSKCWVNWGEAKENIAGIVISGWASGLAGMGH   44
Bacteriocin 31     ATYYGNGLYCNKQKCWVDWNKASREIGKIIVNGWVQHGPWAPR   43
Bacteriocin T8     ATYYGNGLYCNKEKCWVDWNQAKGEIGKIIVNGWVNHGPWAPRR 44
Consensus            YYGNG YCN  KCWV W  A   I  I   GW
(B)
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FIG. 4. Comparison of amino acid sequences of the N termini of class IIa *sec*-dependent bacteriocins. Data for enterocin P and bacteriocin 31 were obtained from the work of Cintas et al. (5) and Tomita et al. (28), respectively.

scribed thus far are enterocin P, produced by *E. faecium* P (5); bacteriocin 31, produced by *E. faecalis* Y1717 (28); and an enterocin P-like bacteriocin produced by *E. faecium* GM-1. The last bacteriocin differs from enterocin P and bacteriocin 31 in being active against gram-positive and gram-negative bacteria (12). The fourth *sec*-dependent bacteriocin belongs to class III and is produced by *E. faecalis* LMG 2333 (20).

The narrow spectrum of activity recorded for bacteriocin T8 is typical of many other class IIa bacteriocins. Bacteriocin 31 also has a narrow spectrum of activity, inhibiting only *Listeria monocytogenes*, *E. faecalis*, and *Enterococcus hirae* (28). Enterocin P and the enterocin P-like bacteriocin have broader spectra of activity. Enterocin P inhibits *L. monocytogenes*, *Staphylococcus aureus*, *Clostridium* spp., *Lactobacillus* spp., *Pediococcus* spp., *Enterococcus* spp., and *Propionibacterium* spp. (5). The decrease in optical density recorded when bacteriocin T8 was added to mid-log-phase cells of *E. faecalis* MDK2 (Fig. 1) indicates that the mode of action is bactericidal, similar to that recorded for enterocin P (5).

Loss of the 7-kb plasmid resulted in a loss of bacteriocin activity, indicating that the genes encoding bacteriocin T8 are located on the plasmid. The structural gene encodes a *sec*-dependent leader peptide with three positively charged amino acids, a number of hydrophobic amino acids, and a cleavage region (Fig. 2 and 4). Two small hydrophobic amino acids are positioned adjacent to the cleavage site (VDA), conforming to the definitions of a typical signal peptide and *sec*-dependent bacteriocins (30). Bacteriocin T8 has a conserved $YGNGX_2CX_4CXV$ motif (Fig. 2 and 4), which is typical of class IIa bacteriocins. As deduced from the DNA sequence, the molecular mass of bacteriocin T8 is 5.1 kDa, similar to the 5.0 kDa recorded for bacteriocin 31 (5) but larger than the 4.6 kDa reported for enterocin P (28). At the amino acid level, bacteriocin T8 is 69% homologous to bacteriocin 31 and 47% homologous to enterocin P (Fig. 4).

The second ORF encodes a potential immunity protein that has a calculated molecular mass of 10.89 kDa (Fig. 2). The gene is located immediately downstream of the structural gene. This is a common feature of lactic acid bacterial bacteriocins (19). The protein is only 50% homologous to the immunity protein of bacteriocin 31. The immunity protein of bacteriocin 31 consists of 94 amino acids and has a molecular mass of 11.0 kDa (5). No homology has been detected with any other immunity proteins listed in GenBank.

The mobilization protein and relaxase mobilization nuclease are presumably involved in the transfer of the plasmid during conjugation. Mobilization genes in enterococci are often located on the same gene cluster as the structural genes of their bacteriocins, as observed for enterocins 1071A and 1071B (4). The genes encoding bacteriocins of enterococci are known to be associated with pheromone-responsive conjugative plasmids (17, 18, 23). Secretion of specific peptide sex pheromones by recipients initiates cell aggregation and conjugation (6, 7). The genes encoding bacteriocin 31 are also located on a pheromone-responsive conjugative plasmid (28).

Although bacteriocin T8 and bacteriocin 31 share certain characteristics, the two peptides are structurally different (69% homology at the amino acid level). The low homology recorded between the signal peptides (27%) and immunity proteins (50%) of the two strains is further proof that bacteriocin T8 is different from bacteriocin 31. Furthermore, the fact that no homology could be detected with any other immunity protein besides that of bacteriocin 31 confirms that bacteriocin T8 is unique.

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