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Biochemical and Genetic Characterization of the Two-Peptide Bacteriocin Enterocin 1071 Produced by *Enterococcus faecalis* FAIR-E 309

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The structural genes for the two-peptide bacteriocin enterocin 1071 (Ent1071) in *Enterococcus faecalis* FAIR-E 309 were cloned. DNA sequence analysis showed that the enterocin 1071A (Ent1071A) peptide of strain FAIR-E 309 differed by two amino acids from the Ent1071A reported for *E. faecalis* BFE 1071 (E. Balla, L. M. T. Dicks, M. Du Toit, M. J. van der Merwe, and W. H. Holzapfel, Appl. Environ. Microbiol. 66:1298–1304, 2000), while the Ent1071B gene encoded identical peptides in these strains. However, resequencing of *ent1071A* from *E. faecalis* BFE 1071 showed that the Ent1071A peptide sequence reported previously was incorrect in two amino acids. Also, *ent1071B* in *E. faecalis* FAIR-E 309 encoded a prepeptide that was three amino acids shorter than that previously reported for *E. faecalis* BFE 1071 Ent1071B. A presumptive immunity gene (*eni1071*) was located downstream of the bacteriocin structural genes. This gene was cloned into the heterologous host *E. faecalis* ATCC 19433 and was shown to confer immunity. A truncated ABC transporter gene was located upstream of the Ent1071 structural genes.

A wide variety of enterocins can be classified as class II bacteriocins (for reviews of bacteriocins, see references 10, 11, 12, and 14); these include enterocin A (1, 15), enterocin B (5, 9), enterocin P (6), enterocin L50 (7), enterocin Q (8), mundticin (3), and enterocin 1071 (Ent1071) (2). Ent1071 is a two-peptide bacteriocin; the enterocin 1071A (Ent1071A) and Ent1071B peptides are both produced as prepeptides which contain a double-glycine type of leader peptide (2), suggesting that these bacteriocins are transported out of the cell by a dedicated transport mechanism consisting of an ABC transporter and an accessory protein. However, the genes necessary for such transport proteins and an immunity gene required for self-protection from the antimicrobial activity of the two-peptide bacteriocin Ent1071 have not been described previously (2).

In this paper we describe biochemical and genetic characterization of Ent1071, a bacteriocin produced by *Enterococcus faecalis* FAIR-E 309 isolated from Argentinian cheese (19), which is identical to Ent1071 produced by *E. faecalis* strain BFE 1071, which was isolated from minipig feces (2). Our studies showed that the peptide sequence previously reported for Ent1071A was incorrect in two amino acids. Also, the Ent1071B prepeptide in this study was shown to be three amino acids shorter than the prepeptide described previously. In addition, we report cloning and expression of a gene involved in immunity to the bacteriocin Ent1071. This study was undertaken as part of a European Union project entitled "Enterococci in Food Fermentations: Functional and Safety Aspects," which, in part, was aimed at identifying novel enterocins that may be used as biopreservatives in cheese production.

The bacterial strains and plasmids used in this study are shown in Table 1. Enterococci were grown in MRS (de Man-Rogosa-Sharpe) broth (Merck, Darmstadt, Germany) at 37°C without agitation, and *Escherichia coli* strains were grown in Luria-Bertani broth (Becton Dickinson, Heidelberg, Germany) at 37°C on a rotary shaker at 250 rpm. The following antibiotics were added as selective agents when appropriate: ampicillin (150 μg ml^{-1}) and erythromycin (200 μg ml^{-1}) for *E. coli* and erythromycin (25 μg ml^{-1}) for enterococci.

Bacteriocin activity was quantified by the critical dilution assay as described previously (9). In this assay, indicator bacteria were inoculated (1%) into MRS soft (0.75%) agar. Bacteriocins were purified by previously established methods (9). Briefly, these methods involved growing *E. faecalis* FAIR-E 309 aerobically for 15 h at 37°C in 2.5 liters of APT broth (Difco, Heidelberg, Germany) with 0.1% Tween 80 added. Cells were removed by centrifugation, and bacteriocin was purified by hydrophobic interaction and cation-exchange chromatography, followed by high-performance liquid chromatography (HPLC), similar to methods described previously (9).

N-terminal sequencing by Edman degradation was done at the Alberta Peptide Institute (Edmonton, Alberta, Canada). Mass spectra of the purified bacteriocins were obtained at the same institute by using a Fisons Micromass VG Quattro instrument (Fisons Instruments, Altrincham, England). Two fractions with bacteriocin activity resulted after HPLC purification, and these fractions were designated Ent1071A and Ent1071B (results not shown). N-terminal amino acid sequencing of the HPLC-purified Ent1071B fraction revealed the

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TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristics[a]	Reference(s) or source
Strains		
E. faecalis FAIR-E 309	*ent1071A*$^+$ *ent1071B*$^+$ *eni1071*$^+$	19
E. faecalis BFE 1071	*ent1071A*$^+$ *ent1071B*$^+$ *eni1071*$^+$	2
E. faecalis ATCC 19433	Ent1071^s, plasmidless	2; this study
E. coli DH5α	F$^-$ *endA1 hsdR17* ($r_K^-m_K^+$) *supE44 thi-1* λ$^-$ *recA1 gyrA96 relA1* Δ(*argF-lacZYA*)*U169* Φ80 d*lacZ*Δ*M15*	BRL Life Technologies, Inc.
E. coli MH1	MC1061 derivative, *araD139 lacX74 galU galK hsr hsm*$^+$ *strA*	4
Plasmids		
pUC118	*lacZ′* Ap^r, 3.2 kb	21
pMG36e	Expression vector, Em^r, 3.6 kb	20
pBFE01	pUC118 containing 424-bp PCR product insert of *ent1071A* and *ent1071B*	This study
pBFE02	pUC118 containing 3.2-kb *Xba*I fragment containing Ent1071 genes	This study
pBFE03	pMG36e containing 407-bp PCR product insert of *eni1071*	This study

[a] Ap^r, ampicillin resistant; Em^r, erythromycin resistant.

following 32-amino-acid sequence: Gly-Pro-Gly-Lys-Trp-Leu-Pro-Trp-Leu-Gln-Pro-Ala-Tyr-Asp-Phe-Val-Thr-Gly-Leu-Ala-Lys-Gly-Ile-Gly-Lys-Glu-Gly-Asn-Lys-Asn-Lys-Trp. A GenBank search indicated that this sequence was identical to that reported previously for Ent1071B (2) except for the last three amino acids, which were reported by Balla et al. (2) but could not be determined in our study. The average molecular masses of Ent1071A and Ent1071B determined by mass spectral analyses in our study were 4,284.66 ± 1.2 and 3,896.15 ± 2.1 Da, respectively (results not shown).

To determine whether *E. faecalis* strains FAIR-E 309 and BFE 1071 produced identical bacteriocin peptides, the structural genes for Ent1071 production were amplified by PCR using total genomic DNA from *E. faecalis* FAIR-E 309 isolated by the methods of Pitcher et al. (16) as the template. The primers used were CFr-1 (5′-TAT AGA ATT CAT ATT TAG GGG GAC CGA TAA-3′) and CFr-2 (5′-TAT ATC TAG AAT ACA TTC TTC CAC TTA TTT TT-3′), which corresponded to the 5′ end of the Ent1071A gene and the 3′ end of the Ent1071B gene as described by Balla et al. (2); primers CFr-1 and CFr-2 contained an *Eco*RI restriction enzyme site and an *Xba*I restriction enzyme site (underlined), respectively. The PCR mixture contained 100 ng of template DNA, each deoxynucleoside triphosphate at a concentration of 200 μM, each primer at a concentration of 25 pM, 1 U of *Taq* DNA polymerase (Amersham Pharmacia), 1× reaction buffer (Amersham Pharmacia), and 1.5 mM $MgCl_2$. DNA amplification was performed for 32 cycles consisting of denaturation at 94°C for 1 min, annealing at 51°C for 1 min, and extension at 72°C for 1 min. The resulting PCR fragment was cloned into the *Eco*RI and *Xba*I restriction enzyme sites of pUC118, resulting in plasmid pBFE01 (Table 1).

Plasmid pBFE01 was used for sequencing to determine the homology between the Ent1071 genes from the two *E. faecalis* strains. DNA was sequenced bidirectionally at MWG Biotech (Ebersfeld, Germany). The DNA sequence for the Ent1071A gene determined in our study differed from that reported by Balla et al. (2) in that it encoded a peptide which differed from the previously reported Ent1071A sequence by two amino acids (Fig. 1 and 2). Thus, instead of the threonine at position 29 and the asparagine at position 38 in the Ent1071A mature peptide reported by Balla et al. (2), the Ent1071A peptide in our study contained an asparagine residue and a lysine residue at these positions, respectively. To verify that these differences in amino acid sequences were not a result of a mismatching error on the part of the *Taq* polymerase used for amplification of the enterocin genes in our study, the genes for Ent1071 production were located and cloned.

Small-scale preparation of plasmid DNA from *E. coli* and enterococci and preparation of electrocompetent *E. coli* and *E. faecalis* ATCC 19433 cells were performed as described previously (9). DNA manipulations and cloning were done as described by Sambrook et al. (18). Recombinant pMG36e plasmid was first transformed into *E. coli* MH1 before it was transformed into *E. faecalis* ATCC 19433 cells. Plasmid pBFE01 was linearized with *Eco*RI, labeled using a digoxigenin-dUTP kit according to the manufacturer's instructions, and used as a probe to detect the location of the Ent1071 genes in *E. faecalis* FAIR-E 309. For this, plasmid DNA from *E. faecalis* FAIR-E 309 was isolated and cut with various restriction enzymes in double- and single-restriction enzyme digestion experiments. Cut plasmid DNA was transferred to a Hybond N+ membrane (Amersham Pharmacia) and hybridized with the probe by using standard methods (18) and the instructions of the manufacturer of the digoxigenin-dUTP kit. A 3.2-kb *Xba*I plasmid DNA fragment from *E. faecalis* FAIR-E 309 was cloned into pUC118 (plasmid pBFE02) for sequencing and further cloning.

DNA sequence analysis revealed that besides the two open reading frames (ORFs) containing the Ent1071 structural genes, a third ORF (*eni1071*) was detected immediately downstream of the Ent1071 gene, while two truncated ORFs were identified on the opposite DNA strand (results not shown). One of these (*repA*) was truncated at the 3′ end of the cloned DNA fragment, and the other (*entT*) was truncated at the 5′ end of the cloned DNA fragment (results not shown).

Nucleotide sequencing revealed that the Ent1071A gene (*ent1071A*) indeed encoded a peptide whose sequence differed from the previously reported (2) sequence for Ent1071A by two amino acids (Fig. 1). However, the amino acid sequences of the leader peptides of Ent1071A from *E. faecalis* strains FAIR-E 309 and BFE 1071 were identical. A probable GGGAA ribosome binding site (RBS) was located 6 bases upstream of the initiation codon of the Ent1071A gene in our study, and possible −10 and −35 promoter sequences were detected upstream of this gene (Fig. 1). A probable RBS

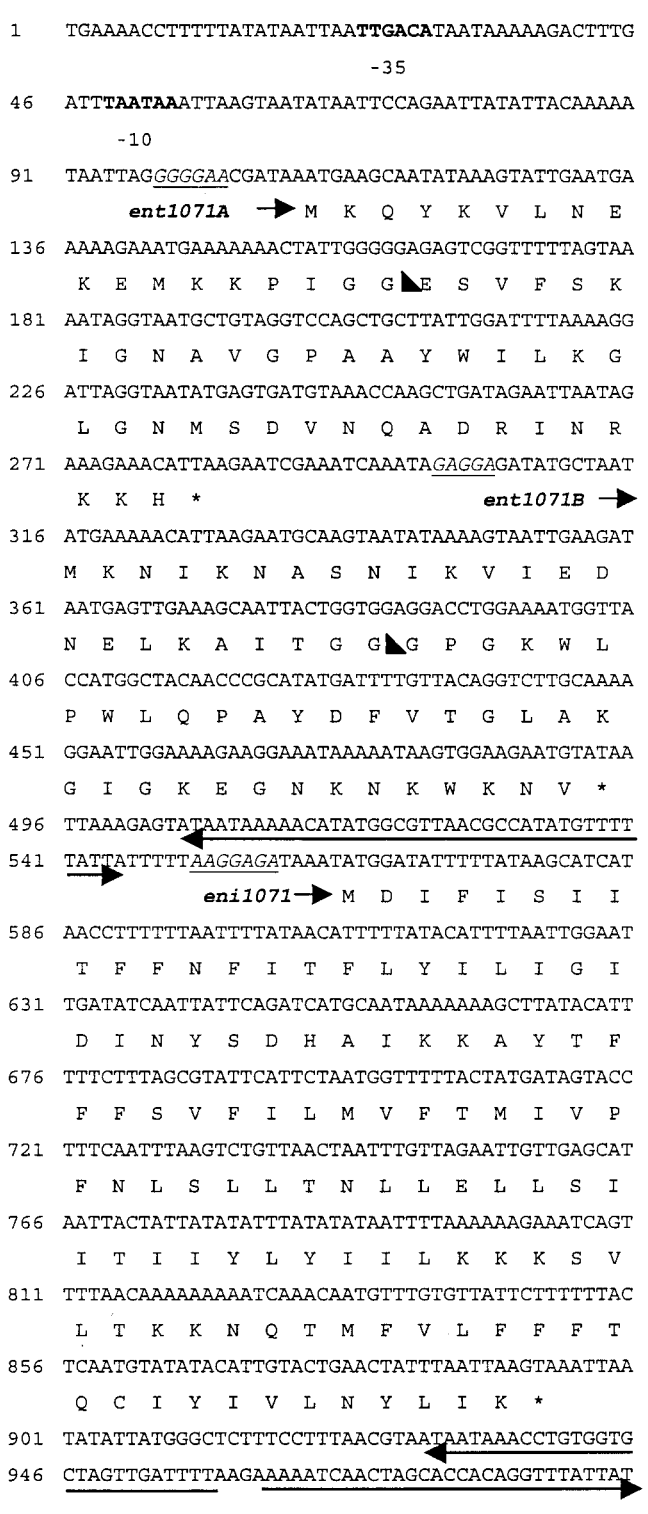

FIG. 1. DNA and deduced amino acid sequences of the Ent1071 structural genes and a gene involved in immunity to Ent1071 from *E. faecalis* FAIR-E 309. The presumptive −10 and −35 promoter sequences are indicated by boldface type, and RBSs are underlined. The direction of transcription of the genes is indicated by arrows. Triangles indicate the processing site for removal of the leader peptides of bacteriocin preproteins. The inverted repeats downstream of *ent1071B* and *eni1071*, which might act as rho-independent transcriptional terminators, are indicated by reversed arrows.

(GAGGA) for *ent1071B* was located 16 bases downstream of the *ent1071B* stop codon, and the initiation codon for *ent1071B* was located 8 bases downstream of this presumptive RBS. The Ent1071B gene also encoded a prepeptide, which contained a 24-amino-acid leader peptide of the double-glycine type. While the mature Ent1071B peptide in our study was identical to that reported by Balla et al. (2), the leader peptide of the double-glycine type was three amino acids shorter than the leader peptide for Ent1071B reported previously (2).

A GenBank search determined that our Ent1071 showed homology only to the Ent1071 reported by Balla et al. (2) and lactococcin G (13). An alignment of the peptide sequences is shown in Fig. 2. The Ent1071A sequence determined in our study showed higher homology (56.4% identity, 59.5% similarity) to the lactococcin Gα sequence than did the Ent1071A sequence determined by Balla et al. (2) (51.2% identity, 54.1% similarity). The molecular masses of Ent1071A and Ent1071B in our study were determined by mass spectrometry to be 4,284.66 and 3896.15 Da, respectively. These values were in good agreement with the theoretically calculated masses of 4,284.88 Da (Ent1071A) and 3,897.49 Da (Ent1071B). However, the molecular mass determined by mass spectrometry for Ent1071A (4,285.16 Da) by Balla et al. (2) did not agree with the theoretically calculated mass for this peptide based on the reported amino acid sequence, which was 4,257.81 Da. Instead, it agreed well with the molecular mass for Ent1071A determined in our study. It appears, therefore, that the amino acid sequence of Ent1071A reported by Balla et al. (2) was erroneous and may have resulted from DNA sequencing errors.

The leader sequence of Ent1071B in the study of Balla et al. (2) was reported to consist of 27 amino acids, while the leader sequence of Ent1071B in this study consisted of only 24 amino acids. The DNA sequence reported by Balla et al. (2) for the region from the stop codon for *ent1071A* to the start of *ent1071B* is identical to the DNA sequence determined in this study except for one additional thymine 29 bases downstream of the Ent1071A stop codon (Fig. 1 and 3). The absence of this thymine in our study shifts the reading frame of *ent1071B* in such a way that there is a stop codon (TGA) 7 bases downstream of the start codon, as indicated for Ent1071B by Balla et al. (2) (Fig. 3). Therefore, the start codon for *ent1071B* in our study could only be the ATG codon that starts 33 bases downstream of the stop codon for *ent1071A* (Fig. 3).

To determine if the discrepancies between the DNA sequences for Ent1071 in our study and in the study of Balla et al. (2) were indeed a result of sequencing errors, the Ent1071 genes were PCR amplified from *E. faecalis* BFE 1071, a strain that came from our institute and that was shared with Balla et al. (2). PCR amplification, cloning, and sequencing of the Ent1071 genes from *E. faecalis* BFE 1071 were performed as described above. Analysis of the DNA sequence from the cloned PCR product showed that the *ent1071A* and *ent1071B* prebacteriocin genes were identical to the genes described in this study, indicating that the DNA sequences of these prebacteriocin genes determined previously (2) must have contained sequencing errors.

An additional ORF (*eni1071*) was detected downstream of *ent1071B*. A possible start codon (ATG) for this gene was located 23 bases downstream of the stop codon for *ent1071B*. However, the presumptive RBS (AAAGAG) associated with

a)

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Enterocin 1071 A, (Balla et al., 2000)      ESVFSKIGNAVGPAAYWILKGLGNMSDVTQADRINRKNH
                                            ESVFSKIGNAVGPAAYWILKGLGNMSDV QADRINRK:H
Enterocin 1071A (this study)                ESVFSKIGNAVGPAAYWILKGLGNMSDVNQADRINRKKH
                                             :    IG:::G  AYW: K::GNMSDVNQA RINRKK
Lactococcin Gα (Nissen-Meyer et al., 1992)   GTWDDIGQGIGRVAYWVGKAMGNMSDVNQASRINRKKKH
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b)

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Enterocin 1071B (Balla et al., 2000; this study)   GPGKWLPWLQPAYDFVTGLAKGIGKEGNKNKWKNV
                                                       WL:W::PAY:F: G::KG  KEGNK:KWKN:
Lactococcin Gβ (Nissen-Meyer et al., 1992)         KKWGWLAWVDPAYEFIKGFGKGAIKEGNKDKWKNI
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FIG. 2. (a) Alignment of the Ent1071A peptide sequences determined by Balla et al. (2) and in this study with the lactococcin Gα peptide sequence; (b) alignment of the Ent1071B peptide sequence determined by Balla et al. (2) and in this study with the lactococcin Gβ peptide sequence determined by Lipman-Pearson alignment. Identical amino acids are shown; similar amino acids are indicated by colons.

this possible start codon (Fig. 1) was considered to be located too far (14 bases) upstream; therefore, the ATG start codon located 66 bases downstream of the stop codon for *ent1071B* was considered more likely to mark the translation start for this protein. This start codon is also preceded by a probable RBS (AAGGAGA) that is spaced more optimally 5 bases upstream of this initiation codon (Fig. 1). This ORF encoded a 110-amino-acid protein with a theoretical molecular mass of 13,083.06 Da and an isoelectric point of 9.278. A palindromic sequence (T1) was detected 9 bases downstream of *ent1071B*, and an inverted repeat (T2) was also detected 33 bases downstream of *eni1071* (Fig. 1). These sequences showed characteristics of possible rho-independent terminators. While T1 was calculated to have a ΔG of -29.3 kcal/mol, the ΔG of T2 was calculated to be -50.8 kcal/mol. The presence of possible terminator sequences both upstream and downstream of *eni1071* may imply that the bacteriocin structural genes and *eni1071* may be located in separate operons.

In a GenBank search, the product of *eni1071* did not show obvious homology to previously reported amino acid sequences. This presumptive Ent1071 immunity gene (*eni1071*) was amplified by PCR using plasmid pBFE02 as the template. Primers CFr-3 (5′-TAT ATC TAG ATG GCG TTA ACG CAT ATG TTT-3′) and CFr-4 (5′-TAT ACT GCA GCG TTA AAG GAA AGA GCC CAT-3′) were complementary to the 5′ and 3′ ends of this gene in pBFE02 and contained *Xba*I and *Pst*I restriction enzyme sites, respectively (underlined). This PCR was performed as described above for Ent1071 gene amplification, except that an annealing temperature of 54°C was used and extension at 72°C was for 40 s. The PCR product was cloned into pUC118 for sequencing to confirm the fidelity of the reaction. In addition, the *eni1071* gene was cloned into the *Xba*I and *Pst*I sites of pMG36e (plasmid pBFE03), and this plasmid was electrotransformed into the Ent1071-sensitive indicator strain *E. faecalis* ATCC 19433 (Table 1). As a negative control, this *Enterococcus* strain was also electrotransformed with pMG36e. For immunity testing, the bacteriocin from *E. faecalis* FAIR-E 309 was partially purified by hydrophobic interaction chromatography of cell-free supernatant as described above for purification (9). Following chromatography, the partially purified bacteriocin was concentrated to a volume of 50 ml by rotary evaporation, and the pH was adjusted to 5.5 with 10 N NaOH. This partially purified bacteriocin was used in critical dilution assays with *E. faecalis* strain FAIR-E 309 containing either plasmid pMG36e or pBFE03 as the indicator. *E. faecalis* ATCC 19433 transformed with pBFE03 was immune to Ent1071 in the critical dilution assay, as no inhibition resulted. In contrast, the negative control, *E. faecalis* ATCC

a)

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                ...AAGAACCATTAAGAATCGAAATCAAATAGAGGAGATATGCTTAATATGAAAAAC.....
ent1071A ⟶ ..K  N  H  *   ent1071B ⟶ RBS     M  L  N  M  K  N  .....
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b)

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                ...AAGAAACATTAAGAATCGAAATCAAATAGAGGAGATATGCTAATATGAAAAAC.....
ent1071A ⟶ ...K  K  H  *                  RBS     M  L  I  *        .....
ent1071A ⟶ ...K  K  H  *   ent1071B ⟶ RBS              M  K  N  .....
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FIG. 3. DNA and deduced amino acid sequences of the 3′ end region of the Ent1071A gene and the 5′ start region of the Ent1071B gene for the Ent1071 locus as reported by Balla et al. (2) (a) and in this study (b). The deduced amino acid sequences following the two presumptive ATG start codons are shown for the Ent1071B gene, as determined in this study (b).

19433 containing plasmid pMG36e, was sensitive to Ent1071 (3,200 activity units/ml) when it was tested with the partially purified bacteriocin. These results showed that *eni1071* encodes a protein involved in immunity towards enterocin 1071.

The ORF *entT* was presumed to encode the ABC transporter gene for Ent1071, as it encodes a protein with homology to ABC transporter proteins of other bacteriocins, such as that of carnobacteriocin B2 (29.4% identity) (17) and that of enterocin A (29.1% identity) (15). These levels of identity are based on the amino acid sequence derived from the truncated *E. faecalis* FAIR-E 309 *entT* gene present in the cloned fragment in this study, and the levels for the entire EntT ABC transporter protein can be expected to be greater. The fact that both Ent1071 peptides are encoded as prepeptides containing a leader sequence of the double-glycine type indicates that they are transported by the products of an ABC transporter gene and an accessory gene, which is generally the case for class II bacteriocins containing this type of leader peptides (12).

Bacteriocin production by *E. faecalis* FAIR-E 309 suggests that this organism could be used as a starter culture for production of traditional cheeses in order to protect against pathogenic bacteria in food, such as *Listeria monocytogenes*. However, before such use, the technological and safety aspects of this strain should be investigated in order to determine its suitability for cheese production.

Nucleotide sequence accession number. The GenBank/EMBL/DDBJ accession number for the sequence reported in this paper is AY063485.

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