**Enterocin C, a Class IIb Bacteriocin Produced by *E. faecalis* C901, a Strain
Isolated from Human Colostrum**

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Running title: Enterocin C from *E. faecalis* C901

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ABSTRACT

Enterocin C (EntC), a class IIb bacteriocin was purified from culture supernatants of *Enterococcus faecalis* C901, a strain isolated from human colostrum. Enterocin C consists of two distinct peptides, named EntC1 and EntC2, whose complementary action is required for full antimicrobial activity. The structural genes *entC1* and *entC2* encoding enterocins EntC1 and EntC2, respectively, and that encoding the putative immunity protein (*EntCI*) are located in the 9-kb plasmid pEntC, harboured by *E. faecalis* C901. The N-terminal sequence of both antimicrobial peptides revealed that EntC1 is identical to Ent1071A, one of the two peptides that form enterocin 1071 (Ent1071), a bacteriocin produced by *E. faecalis* BFE 1071. In contrast, EntC2 presents the non-polar alanine residue at position 17 (Ala₁₇) instead of the polar threonine residue (Thr₁₇) in Ent1071B, the second peptide constituting Ent1071. In spite of peptide similarities, EntC differs from Ent1071 in major aspects, including the complementary activity among its constitutive peptides and its wider inhibitory spectrum of activity. Different amphiphilic α -helical conformations between EntC2 and Ent1071B could explain both, acquired complementary activity and increased antimicrobial spectrum.

INTRODUCTION

Colostrum and breast milk protect the newborn against infectious diseases, being this effect due to the combined action of a variety of protective factors present in these biological fluids, such as immunoglobulins, immunocompetent cells, antimicrobial fatty acids, polyamines, fucosylated oligosaccharides, lysozyme, lactoferrin, and antimicrobial peptides, which inactivate pathogens individually, additively, and synergistically (27). In addition, breast milk is an excellent and continuous source of commensal and potentially probiotic bacteria to the infant gut, including staphylococci, streptococci, and other lactic acid bacteria (LAB) (23, 33, 35). Some of the LAB strains isolated from this biological fluid have the ability to inhibit the growth of a wide spectrum of pathogenic bacteria by competitive exclusion and/or through the production of antimicrobial compounds, such as organic acids, hydrogen peroxide or the bacteriocin nisin, a lactococcal bacteriocin (6, 34, 43).

Bacteriocins are antimicrobial compounds of proteinaceous nature with inhibitory activity against microorganisms that usually are closely related to the producing bacteria. In recent years, bacteriocins from LAB have been extensively studied due to their potential as food preservatives. The ability to produce such antimicrobial peptides is wide spread among enterococci (40), a property that makes them attractive to the food industry; in fact, several enterococcal strains with histories of safe use are included as components of starter, bioprotective or probiotic cultures (20).

Enterococci form an essential part of the indigenous human microbiota soon after birth, being *Enterococcus faecium* and *Enterococcus faecalis* the most common species in human mucosal surfaces (16, 26, 46). On the other hand, some enterococcal strains can behave as opportunistic pathogens and cause nosocomial infections especially in patients with underlying

diseases (29). Moreover, enterococci are noted for their capacity to exchange genetic information, including antibiotic resistance genes, by conjugation (12, 15).

In this study, a two-peptide bacteriocin (enterocin C) produced by *E. faecalis* C901, a strain originally isolated from human colostrum provided by a healthy woman, was biochemical and genetically characterized. In addition, this strain was screened for the presence of potential virulence determinants and for its sensitivity to several clinically-relevant antibiotics.

MATERIALS AND METHODS

Bacterial strains and media. *E. faecalis* C901 and all the bacterial strains used as indicators in this study (Tables 1 and 2) were grown routinely in MRS medium (Oxoid, Basingstoke, Hampshire, England) at 37°C, with the exception of *Actinomyces* sp., *Enterococcus gallinarum*, *E. saccharolyticus*, *Escherichia coli*, *Staphylococcus* sp. and *Streptococcus* sp., which were grown in Brain Heart Infusion (BHI) medium (Oxoid). All the strains from human sources (Table 1) belonged to our own collection while those from food origin were obtained from different bacterial collections (Table 2). They were maintained as frozen stocks at –80°C in MRS or BHI (Oxoid) plus 20% (vol/vol) glycerol.

Bacteriocin assays. Bacteriocin activity in cell-free supernatants (CFSs) from exponential and stationary-phase broth cultures (8 and 16 h, respectively) of *E. faecalis* C901 was assayed by using the agar drop diffusion test as described previously (31), using the strains listed in Tables 1 and 2 as indicator microorganisms. Subsequently, *E. faecalis* L1443 was used as the indicator strain throughout the whole process of enterocin C purification and, also, to investigate the complementary activity of the two peptides (EntC1 and EntC2) that integrate enterocin C. For the late purpose, 5-μl aliquots of purified EntC1 were mixed with equal

1 volumes of purified EntC2, and the mixes were assayed for antimicrobial activity by the agar
2 drop diffusion test. Five- μ l aliquots of each purified peptide were separately assayed in order to
3 compare the results. Quantification of complementary activity among peptides EntC1 and
4 EntC2 of enterocin C was carried out by the microtiter plate assay system (21). The inhibitory
5 activity was expressed as bacteriocin units per millilitre (BU/ml) as described previously (31).

6
7 **Bacteriocin purification.** All the purification steps were carried out at room
8 temperature, and all of the chromatographic equipment and media were purchased from
9 Amersham Biosciences Europe GmbH (Freiburg, Germany). Peptides EntC1 and EntC2 were
10 purified from a 2-L culture of *E. faecalis* C901. After 24 h at 37°C without shaking, cells were
11 removed by centrifugation at $10,000 \times g$ for 10 min at 4°C and, then, the bacteriocin was
12 purified from the CFS using the procedure described by Maldonado *et al.* (30). Briefly, the CFS
13 was precipitated with ammonium sulfate, desalted, and consecutively applied to cation-
14 exchange and hydrophobic-interaction columns. Finally, samples were subjected to C₂/C₁₈
15 reverse-phase chromatography in FPLC (RPC-FPLC). Fractions showing inhibitory activity
16 after the C₂/C₁₈ reverse-phase column were pooled and subjected to several runs until both
17 EntC1 and EntC2 peptides were purified to homogeneity.

18
19 **SDS-PAGE.** During the purification process, the C₂/C₁₈ reverse-phase column fractions
20 were analyzed in duplicate by SDS-PAGE using an 18% acrylamide resolving gel. After
21 electrophoresis at 100 mV for 2 h, one gel was silver stained (52) while the other was used to
22 detect inhibitory activity in an overlay assay with *E. faecalis* L1443 as the indicator strain (8).

23
24 **N-terminal amino acid sequence and mass spectrometry.** The N-terminal amino acid
25 sequences of purified EntC1 and EntC2 peptides were determined by automated Edman

degradation with a Beckman LF3000 sequencer/phenylthiohydantoin amino acid analyzer (System Gold, Beckman, Fullerton, CA). Molecular mass of the peptides was determined by Matrix Assisted Laser Desorption/Ionization-Time of Flight Mass Spectrometry (MALDI-TOF). These analysis were performed by Dr. Silvia Bronsoms (Servei de Proteòmica i Bioinformàtica, Universitat Autònoma, Barcelona, Spain).

PCR sequencing and location of the enterocin C structural and immunity genes.

The primer pair 1071A-for (5'- ATGAAGCAATATAAAGTATTGAATG -3') and 1071I-rev (5'- TTACTTAATTAAATAGTTCAGTACA -3') were designed on the basis of the genes encoding Ent1071A (*ent1071A*) and Ent1071B (*ent1071B*) and their immunity protein (*entI*) (GenBank accession number: AF458698). DNA was amplified in 25- μ l reaction mixtures containing 2.5 mM Mg Cl₂, 1 $\times$ reaction buffer, 200 μ M concentrations of each of the deoxynucleotides triphosphates (dNTPs), 1 μ M of each of the primers, and 1,25 U of *Taq* DNA polymerase (Ecotaq; Ecogen, Barcelona, Spain). Amplification included denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 1 min, polymerization at 72°C for 1 min, and a final polymerization step at 72°C for 5 min.

The amplified fragment was excised from a 0.7% agarose gel, purified using the Nucleospin® Extract II kit (Macherey-Nagel, Düren, Germany), and both strands were sequenced using primers 1071A-for and 1071I-rev at the Genomics Unit of the Universidad Complutense (Madrid, Spain).

To locate the genes encoding EntC1 and EntC2 peptides, PCR assays were performed as described above, using plasmid or chromosomal DNA from *E. faecalis* C901 as templates. Plasmid DNA was extracted as described by Anderson and McKay (2) while total genomic DNA was isolated using the QIAmp tissue kit as recommended by the manufacturer.

1 **PCR screening of virulence and vancomycin-resistance (*van*) genes in *E. faecalis***

2 **C901.** A novel multiplex PCR assay was designed to detect the presence of the potential
 3 virulence determinants *gelE*, *cylA*, *efaA_{fs}*, *eps_{fs}*, *agg₂*, *ccf*, *cpd*, *cad*, and *cob* in *E. faecalis* C901
 4 using the primer pairs previously proposed by Eaton and Gasson (15). PCR amplifications were
 5 performed in 25- μ l reaction mixtures containing 3 mM Mg Cl₂, 1.5 $\times$ reaction buffer, 400 μ M
 6 of each deoxynucleotide triphosphate (dNTP), 0.2 μ M of each primer, and 2,5 U of *Taq* DNA
 7 polymerase (Ecotaq). A colony suspension in deionized water (5 μ l) was used as DNA
 8 template. PCR conditions included initial denaturation at 94°C for 5 min, followed by 30 cycles
 9 of denaturation at 94°C for 1 min, annealing at 51°C for 30 s, elongation at 72°C for 1 min 30
 10 s, and a final extension at 72°C for 5 min. *E. faecalis* P229 (15) was used as a positive control.

11 PCR for detection of *vanA* and *vanB* genes were carried out according to Dutka-Malen
 12 *et al.* (14) and Ramos-Trujillo *et al.* (49), respectively. *E. faecium* BM4147 (VanA⁺) and *E.*
 13 *faecalis* V583 (VanB⁺) were used as positive controls.

15 **Computer analysis of DNA sequences.** The Clone Manager Professional Suite
 16 software (version 6.00) was used for DNA analysis. The WinPep program (available at
 17 <http://www.ipw.agrl.ethz.ch/~lhennig/winpep.html>) was used for physico-chemical analysis of
 18 peptides (isoelectric point, molecular weight and hydrophathy plot) (24). Edmunson α -helical
 19 wheel (51) was plotted with the Helical Wheel Applet program
 20 (<http://cti.itc.virginia.edu/~cmg/Demo/wheel/wheelApp.html>).

22 **Nucleotide sequence accession number.** The DNA sequence presented in this article
 23 has been deposited in the GenBank database under the accession number EU862242.

Hemolytic activity and susceptibility to antibiotics. Hemolytic activity of *E. faecalis* C901 was determined on Columbia agar plates supplemented with 5% horse blood (COH, BioMerieux) and incubated for 72 h at 37°C. Minimal inhibitory concentrations (MICs) for several antibiotics commonly used against enterococcal infections were determined by a microdilution method using the Sensititre plates Staenc1F (Trek Diagnostic Systems, Cleveland, OH) following the manufacturer's instructions. Briefly, colonies from solid media were suspended in saline solution to reach a 0,5 McFarland turbidity. Fifty µL of this suspension were transferred to a 10 ml tube of Mueller–Hinton broth (bacterial concentration of ca. 10⁶ cfu/ml) and, finally, 100 µl of this suspension were inoculated into each plate well. Antibiotics analyzed were amoxycillin/clavulanic acid (AUG), ampicillin (AMP), chloramphenicol (CHL), ciprofloxacin (CIP), clindamycin (CLI), erythromycin (ERY), fosfomicin (FOS), gentamicin (GEN), imipenem (IMI), mupirocin (MUP), nitrofurantoin (NIT), linezolid (LNZ), oxacillin (OXA), penicillin (PEN), quinupristin/dalfopristin (Q/D), rifampin (RIF), streptomycin (STR), teicoplanin (TEI), trimethoprim/sulfamethoxazole (SXT), tetracycline (TET) and vancomycin (VAN). When available, the National Committee for Clinical Laboratory Standards (NCCLS; 2002) were used for determination of sensitivity/resistance (38).

RESULTS

Antimicrobial activity of *E. faecalis* C901. CFSs obtained from exponential and stationary-phase *E. faecalis* C901 cultures showed antimicrobial activity against many indicator strains used in this study, including species both related and non-related to the producing strain, such as *A. neuui*, *E. faecalis*, *E. faecium*, *F. hominis*, *L. lactis*, *L. paracasei*, *L. mesenteroides*, *P. acnes*, *S. caprae*, *S. epidermidis*, *S. anginosus* and *S. intermedius* (Tables 1 and 2). It is

1 noteworthy that all but one *E. faecium* and *E. faecalis* strains tested as indicators were
2 inhibited, including other bacteriocin-producing strains.

3
4 **Purification of EntC1 and EntC2 peptides.** Similarly to many LAB bacteriocins, the
5 compound responsible for the antimicrobial activity produced by *E. faecalis* C901 precipitated
6 in the presence of ammonium sulfate and showed a cationic and highly hydrophobic nature.
7 Evidence that two peptides were involved in enterocin C activity was obtained after the first
8 run in the C₂/C₁₈ reverse-phase column, when the agar drop diffusion test revealed that
9 maximal antimicrobial activity was coincident with fractions in which two distinct absorbance
10 peaks overlapped (not shown). Several additional runs were necessary to obtain fractions
11 containing pure peptides, which were named EntC1 and EntC2, according to the order they
12 eluted from the column. Fractions containing EntC1 showed little or no inhibitory activity in
13 contrast to that displayed by fractions containing EntC2. SDS-PAGE gels showed that both
14 peptides had been purified to homogeneity and displayed similar molecular weight (ca. 2.5
15 kDa; Fig. 1A). When SDS-PAGE gels were assayed for antimicrobial activity, both peptides
16 were active against the indicator strain *E. faecalis* L1443, being the activity displayed by EntC2
17 notably higher (Fig. 1A). In addition, a small inhibition halo was observed in the zone between
18 the bands corresponding to EntC1 and EntC2, which resulted from diffusion and mixing of
19 both peptides, suggesting again their complementary nature (Fig. 1A). Complementary assays
20 revealed that the bacteriocin activity of the two-peptide mix was greater than that observed for
21 each separated peptide (Fig. 1B). More specifically, when bacteriocin activity of eluted
22 fractions containing pure peptides was titrated, EntC1 showed no or just residual activity,
23 meanwhile EntC2 showed 200 BU/ml. However, when EntC1 and EntC2 were combined in the
24 same proportion, bacteriocin activity was 25600 BU/ml, ca. 128 times more than EntC2

assayed alone. These results confirmed the existence of complementary activity between the peptides EntC1 and EntC2.

Partial amino acid sequence and mass spectrometry of EntC1 and EntC2. Partial amino acid sequencing of EntC1 and EntC2 showed that the 10 N-terminal amino acids shared 100% homology with the first 10 N-terminal amino acids of mature enterocin 1071A (Ent1071A) and 1071B (Ent1071B), respectively, from *E. faecalis* BFE 1071. Mass spectrometry analysis (MALDI-TOF) rendered molecular masses of 4,284 Da for EntC1 and 3,867 Da for EntC2 (Fig. 2). The MW of mature Ent1071A and Ent1071B were 4,285 and 3,899 respectively (4). These results suggested that the amino acid sequence of peptide EntC1 could be identical to that of Ent1071A while the differences in the molecular masses between EntC2 and Ent1071B could be attributable to a variation in their amino acid sequence.

Genetic analysis and DNA sequencing of enterocin C structural genes. In order to analyse the DNA sequence encoding enterocin C, a 786-bp DNA fragment was amplified with primers 1071A-for/1071I-rev using DNA from pENTC, a 9-kb size plasmid extracted from *E. faecalis* C901, as template. Sequencing of this DNA fragment revealed the presence of three open reading frames (ORFs) which shared high homology with the structural genes encoding enterocins 1071A (*ent1071A*) and 1071B (*ent1071B*) and their immunity protein 1071I (*ent1071I*). Detailed analysis of this sequence showed that *entC1* and *entC2* encoded two peptides of 57 and 62 amino acids, respectively. Both peptides contain leader sequences of the double-glycine type that, upon processing, give rise to mature peptides of 39 and 35 amino acids, respectively, whose deduced molecular masses (4,284 and 3,899 Da respectively) were coincident with those obtained experimentally for EntC1 and EntC2 peptides through mass spectrometry (Fig. 3). As expected, the deduced amino acid sequence of mature peptide EntC1

was identical to mature enterocin 1071A. However, the amino acid sequence deduced for mature EntC2, differed in one amino acid with that of mature enterocin 1071B (Fig. 3). More specifically, EntC2 contains an alanine residue in position 17 (A₁₇) instead of the threonine residue of the peptide Ent1071B at the same position (T₁₇), due to the substitution of a single nucleotide (GCA in *entC2*, while ACA in *ent1071B*). The molecular mass predicted for the EntC2 peptide corresponded exactly with that obtained by MALDI-TOF, confirming the A₁₇ of enterocin C901. The double-glycine leader sequences of both the EntC1 and EntC2 peptides were identical to those described for Ent1071A and Ent1071B, respectively.

Downstream of *entC2*, a third ORF named *entCI* was found. This ORF encodes a putative protein of 125 amino acid residues with a theoretical pI of 9.4, and a MW of 14,866 Da, which shares 100% homology with 1071I, the protein that putatively confers immunity to enterocin 1071A and 1071B. Finally, we found that the whole operon encoding enterocin C production and immunity is harboured by a 9-kb plasmid in *E. faecalis* C901, which was designated pENTC.

Virulence determinants, *van* genes and sensitivity to antibiotics of *E. faecalis* C901.

The genes *gelE*, *efaA_{fs}*, *eps_{fs}*, *agg2*, *ccf*, *cpd*, *cad* and *cob* were present in *E. faecalis* C901 (Fig. 4) but *cylA* was not detected. Haemolysis was not observed on COH plates. On the other hand, genes *vanA* and *vanB*, conferring resistance to vancomycin, were absent in this strain. MICs of selected antibiotics are shown in table 3. In summary, *E. faecalis* C901 was sensitive to most of the antibiotics tested with the exceptions of tetracycline and quinupristin-dalfopristin.

DISCUSSION

To our knowledge, this is the first report describing the isolation of a bacteriocin-producing LAB from human colostrum. Our results clearly indicate that enterocin C belongs to

the class IIB bacteriocins (39) and, therefore, consists of two different peptides, EntC1 and EntC2, whose combination is necessary to obtain full bacteriocin activity.

Enterocin C is nearly identical to enterocin 1071, a bacteriocin previously described which has been purified from two independent enterococcal strains: *E. faecalis* BFE1071 and *E. faecalis* FAIR E-309 (4, 19). In fact, the amino acid sequence of peptide EntC1 is identical to that of Ent1071A, while EntC2 differed only in one amino acid from enterocin 1071B (A₁₇ and T₁₇, respectively). In contrast with the results obtained in this work with peptides EntC1 and EntC2, whose activity is clearly complementary, it has been described that enterocin 1071A acts independently from enterocin 1071B (5). In addition, it has been highlighted that the absence of activity against lactococci is a typical feature of enterocins 1071A and 1071B (4, 5, 19). However, enterocin C shows inhibitory activity against *L. lactis* strains including *L. lactis* IL1403 (Tables 1 and 2), a strain resistant to enterocin 1071 (4). In the same manner, enterocin C was active against *L. sakei* NCFB 2714 but not against *L. salivarius* NCFB 2747, two strains which were resistant and sensitive, respectively, to enterocin 1071 (4). Therefore, the inhibitory spectrum of enterocin C appears to be quite distinct to that of enterocin 1071.

A single amino acid change in one of the peptides which compose class IIB bacteriocins could be responsible for determining the specificity of the target strain. Lactococcin G (composed by LcnG α plus LcnG β) and lactococcin Q (composed by LcnQ α plus LcnQ β) are two homologous (88% identity) two-peptide bacteriocins whose inhibitory activity is limited to lactococcal strains (42, 55). The peptide LcnG α differs in six amino acids with LcnQ α while peptide LcnG β differs in only three amino acids with LcnQ β . Both bacteriocins are also similar to enterocin 1071 (57 and 59 % identities to lactococcins G and Q, respectively) (44). Zendo *et al.* (55) suggested that the different amino acid residues between LcnG β and LcnQ β might be involved in the intensity of the antibacterial activity rather than in determining the specificity for target cells. On the other hand, since enterocin 1071 was not active against lactococcal

1 strains, they proposed that the amino acid residues that differ between lactococcins and
2 enterocin 1071 might be involved in the specific recognition of target cells. Taking into account
3 these observations, it could be possible that the amino acid residue at position 17 (Ala) in the
4 peptide EntC2 of enterocin C is located in a zone involved in specificity determination.

5 In a recent study, Oppegård *et al.* (45) analysed bacteriocins lactococcin G and
6 enterocin 1071 by site-directed mutagenesis. Their results suggested that the β peptide of each
7 bacteriocin (LcnG β and Ent1071B) is important at determining target cell specificity, specially
8 the N-terminal residues. Besides, the C-terminal residues might be involved in specific
9 interaction with the cognate α peptide (LcnG α and Ent1071A, respectively). Thus, EntC2
10 represents an Ent1071B natural variant whose study in combination with EntC1 and/or other
11 similar two-peptide bacteriocins, such as lactococcins G and Q, could serve to elucidate the
12 mode of action of these antimicrobial peptides as well as determine how target cell specificity
13 is achieved. Actually, one single amino acid difference between EntC2 and Ent1071B (Ala₁₇
14 and Thr₁₇, respectively) appears to be responsible for the high inhibitory activity of enterocin C
15 against lactococci compared with the lack of activity of enterocin 1071. This amino acid
16 change could also be responsible for the considerable enhancement in antimicrobial activity
17 due to complementarity observed in enterocin C, as this change is not neutral, for a polar amino
18 acid (Thr in Ent1071B) has been substituted by a non-polar one (Ala in EntC2). On the other
19 hand, when Ent1071A and Ent1071B peptides were obtained after site-directed mutagenesis
20 from LcnG α and LcnG β , respectively, and heterologously expressed in *L. sake* Lb706, the
21 mixture was active against lactococci (45). This result could indicate that the actual way by
22 which a specific bacteriocin producer strain synthesizes a bacteriocin plays an extremely
23 important role in its final power and spectrum of activity. Apart from particular amino acid
24 changes that could determine important conformational modifications, facts such as the relative
25 amounts produced of each complementary peptide by a specific producer strain could explain

1 why very similar two-peptide bacteriocins, such as enterocin C and enterocin 1071, can differ
2 in their activity spectrum and complementary effect between their constitutive peptides.

3 Amphiphilic α -helical conformations are found in several pore-forming antimicrobial peptides
4 (48). Class IIb bacteriocins, as lactococcin G, are thought to form pores due to the amphiphilic
5 character or their constituting peptides, which can adopt an α -helix conformation (22, 45).

6 Attending to their amino acid sequence, amphiphilic α -helical motifs of enterocin C and 1071
7 are represented as an Edmunson α -helical wheel (53) in figure 5. As it is shown in this figure,
8 the non-polar Ala₁₇ in EntC2 is in the place of the polar Thr₁₇ in Ent1071B just in the
9 hydrophilic side of the α -helix. This change in the polarity of that region could facilitate
10 dimerization with EntC1 or interaction with the membrane of target cells. Thus, it could ease
11 pore formation in the membranes of sensitive strains and/or increase the sensitivity of these. It
12 is noticeable that the amphiphilic regions of lactococcins G β and Q β are highly similar to that
13 of EntC2 when represented as an α -helical wheel (Fig. 5), despite the differences in their
14 aminoacid sequences (Fig. 3).

15 *E. faecalis* C901, isolated from human colostrum, produces a bacteriocin that is almost
16 identical to enterocin 1071 produced by *E. faecalis* BFE 1071 and Fair-E 309, two strains that
17 were isolated from mini pig faeces (4) and cheese (19), respectively. Enterocin C, as as it is
18 with enterocin 1071 (5), is encoded by a plasmid that we have named pENTC and which is
19 currently under sequencing. The extremely efficient plasmid conjugation system of *E. faecalis*
20 (25) can explain the widespread of these highly homologous enterocins among different strains.
21 In fact, *E. faecalis* C901 has shown to possess the genes *cpd*, *cob*, *ccf*, *cad* encoding sex
22 pheromones which could facilitate conjugation with other *E. faecalis* strains. In addition, this
23 strain harbours the *gelE*, *efaA_{fs}*, *eps_{fs}*, and *agg₂* genes but it lacked the *cylA* gene. The presence
24 of the cited genes seems to be widespread among *E. faecalis* strains, including those with a
25 long history of safe use in the food industry (15, 18). Despite the apparently “dualistic” nature

1 of enterococci, the incidence of virulence determinants and/or any other factor of clinical
2 significance, such as the antibiotic resistance pattern or the gene transfer potential, appear to be
3 strain specific among isolates studied so far. Vancanneyt *et al.* (54) compared the genotypes of
4 strains of the related specie *E. faecium* from human, animal and food origin and found that all
5 human isolates involved in clinical infection fell into a well defined subgroup, suggesting that
6 there could be a common genetic basis for strains associated with human disease. Pillai *et al.*
7 (47) also suggested that virulent subpopulations of *E. faecalis* may exist. Therefore, the safety
8 of any enterococcal strain should be individually evaluated.

9 Enterococci are among the predominant bacteria in human milk of healthy women (23,
10 33, 35) and in faeces of healthy breastfed neonates (1, 51), which suggests that they may play
11 important biological functions. The roles of enterococci in human hosts, both in health and
12 disease, are far from clear, but the inclusion of enterococcal strains among the microorganisms
13 that will be studied in the frame of the human microbiome project may provide new clues in the
14 next years.

18 ACKNOWLEDGMENTS

19 This work was supported by the FUN-C-FOOD (Consolider-Ingenio 2010), AGL2006-00763
20 and AGL2007-62042 projects from the Ministerio de Educación y Ciencia (Spain). A. M. was
21 the recipient of a post-doctoral grant (MEC-Fulbright program) from the Ministerio de
22 Educación y Ciencia (MEC).

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LEGENDS OF THE FIGURES

Legend of Figure 1. A) Left panel, silver stained SDS-PAGE corresponding to EntC1 and EntC2 purified peptides as indicated; right panel, bioassay of the same EntC1 and EntC2 samples using *E. faecalis* L1443 as the sensitive strain, showing the corresponding inhibition zones; the arrow indicates the inhibition halo produced by the complementary action of both peptides; on the left of the panels, molecular weight markers. B) Spot-on-lawn test showing the inhibitory activity of EntC1 and EntC2 purified peptides and a mixture of them to demonstrate their complementary action; *E. faecalis* L1443 was used as the sensitive strain; the bar represents 10 mm.

Legend of Figure 2. MALDI-TOF mass spectra analysis corresponding to EntC1 and EntC2 peptides. a.i., arbitrary intensity. Estimated molecular weights are indicated on top of the corresponding mass spectra peaks.

Legend of Figure 3. Amino acid sequence of EntC1 and EntC2 mature peptides deduced from their coding DNA sequences and alignment with homologous two-peptide bacteriocins Enterocin 1071 (Ent1071A + Ent1071B), Lactococcin G (LcnG α + LcnG β) and Lactococcin Q (LcnQ α + LcnQ β). Identical amino acid residues are boxed. For LcnQ, only those amino acid residues which are different from LcnG are indicated; among these, residues in italics are those identical to EntC1. Amino acid residues which are different in EntC2 and 1071B are underlined. Theoretical (t) and experimental (e) molecular weights are indicated.

Legend of Figure 4. Agarose gel electrophoresis of multiplex PCR analysis of virulence determinants. Lanes: M, molecular weight marker; the actual sizes are indicated on the left;

C901, result using total DNA from *E. faecalis* C901 as the template; P229, result using total DNA from *E. faecalis* P229 as the template, used as the positive control in the PCR reactions. Virulence genes corresponding to each specific PCR-amplified DNA band are indicated at the right side.

Legend of Figure 5. Edmundson α -helical wheel representation of the amphiphilic regions in enterocins EntC2 and Ent1071B, and lactococcins LcnG β and LcnQ β . In all peptides the amphiphilic region starts at the amino acid residue 8 and ends with the amino acid residue 25. Polar and nonpolar amino acid residues are shown as white or shaded circles, respectively. Residues which are different in enterocin EntC2 with respect to enterocin Ent1071B, and in lactococcin LcnQ β to lactococcin LcnG β are marked with boxes.

Table 1. Inhibitory spectrum of cell-free supernatants from *Enterococcus faecalis* C901 against bacterial strains from human origin.

Bacterial species	Strain ^a	Sensitivity ^b
<i>Actynomyces neuui</i>	FR1543	++
	P1543	+
<i>Bifidobacterium longum</i>	H1542	-
<i>Enterococcus faecalis</i>	C301, L1443	++++
	EV1444, FR1441, FR1542	+++
	H1441, HK223, LAM43	
	MA006, SC1541	
	C1002, EV1542, L1543	++
	LA1442, LV123, M1441	
	M1541, P1441a, SC1442	
<i>Enterococcus faecium</i>	C656	+++
<i>Enterococcus gallinarum</i>	HU521	-
<i>Enterococcus sacharolyticus</i>	HU522	-
<i>Escherichia coli</i>	FR1545	-
<i>Facklamia hominis</i>	EV1443	++
<i>Lactobacillus fermentum</i>	Lc40	-
<i>Lactobacillus gasseri</i>	EV1461, LA2441, Lc9, Lc23	-
<i>Lactobacillus paracasei</i>	C1351, C1352	++++
<i>Lactobacillus reuteri</i>	EV1763, LA1746	-
<i>Lactobacillus rhamnosus</i>	FR1762	-
<i>Lactobacillus salivarius</i>	HN6	-
<i>Leuconostoc mesenteroides</i>	C1353	++++
<i>Propionibacterium acnes</i>	P1544, SC1441, SC1544b	+++
<i>Propionibacterium avium</i>	H1544b	-
<i>Propionibacterium granulosum</i>	C1441	-
<i>Staphylococcus caprae</i>	FR1541a	+
<i>Staphylococcus epidermidis</i>	EV1541	-
	C1541 EV1441 FR1444	-
	FR1541b L1442 L1544	-
	L1546 M1564 P1441b	-
	P1541 SC1444b	-
<i>Streptococcus anginosus</i>	EV1442	+++
	FR1442, L1441, LA1441	-
<i>Streptococcus intermedius</i>	LA1443	+
<i>Streptococcus parasanguinis</i>	L1541	-

^a Abbreviations: C, colostrums; H, faeces; EV, vaginal exudates; FR, rectal frotis; M, meconium; L, breast milk; LA, amniotic liquid; P, skin; SC, umbilical cord blood.

^b Sensitivity to cell-free supernatants of *E. faecalis* C901, assayed by the spot-on-lawn method. Sensitivity scale: + (<9mm), ++ (10-11 mm), +++ (11-12mm), and ++++ (12-14 mm), reflect the degree of sensitivity according to the diameter of the inhibition halo showed in brackets; -, resistant.

Table 2. Inhibitory spectrum of *Enterococcus faecalis* C901 against several bacteriocin producing and non-producing strains.

Bacterial species	Strain (bacteriocin) ^a	Source ^b	Sensitivity ^c	Reference
<i>Enterococcus faecium</i>	LP6T1a (L50A+L50B)	CIG	+++	(11, 17)
	CTC492 (EntA+EntB)	CTC	+	(3, 9, 41)
	P13 (EntP)	FVM	+++	(10)
<i>E. faecalis</i>	EFS2 (AS-48)	UG	+++	(30, 37)
	OG1-X (AS-48)	UG	++++	(37)
	AE9, AE12, AE23 (Munt)	HRM	++	(7)
	PB1 (bac+)	HRM	+++	
	EF1	TNO	++++	
	CNRZ 135,136, 137	INRA	+++	
	CNRZ 34 (bac+)	INRA	-	
	BM4100WT	TNO	+++	
<i>Lactobacillus acidophilus</i>	NCDO 1748	NCDO	-	
	ATCC 4356	TNO	-	
<i>L. brevis</i>	LB9	UV	-	
<i>L. casei</i>	NCDO 161	NCDO	-	
<i>L. curvatus</i>	NCFB 2739	NCDO	-	
<i>L. delbrueckii</i>	ATCC 11842	IPLA	-	
<i>L. fermentum</i>	ATCC1493	ATCC	-	
	NCDO1750	NCDO	-	
<i>L. hilgardii</i>	LB76	UV	-	
<i>L. pentosus</i>	LPCO10 (plnS)	CIG	-	(28)
	128/2	CIG	-	
	CECT 4023	CECT	-	
<i>L. plantarum</i>	NC8 (plnC8+ plnEF + plnJK)	MATFORSK	-	(13, 31, 32)
	LB6	UV	-	
	CECT748	CECT	-	
<i>L. reuteri</i>	DSM 20016	TNO	-	
<i>L. salivarius</i>	NCFB 2747	TNO	-	
<i>L. sakei</i>	NCFB 2714	TNO	++	
<i>Lactococcus lactis</i>				
<i>subsp. cremoris</i>	MG1363	CIT	+	
	CNRZ117	INRA	-	
<i>subsp. diacetylactis</i>	IPLA838	IPLA	+++	
<i>subsp. lactis</i>	IL1403	INRA	++++	
	IPLA972 (lcn972)	IPLA	++	(36)
<i>Pediococcus parvulus</i>	P339	UV	-	
<i>P. pentosaceus</i>	FBF63, P56	TNO	-	
<i>Streptococcus thermophilus</i>	ST20	TNO	-	

^aBacteriocin produced is shown in brackets.

^b Abbreviations: ATCC, American Type Culture Collection (Rockville, Md.); CECT, Colección Española de Cultivos Tipo (Universidad de Valencia, Burjasot, Spain); CIG, Colección Instituto de la Grasa (Sevilla, Spain); CIT, Cranfield Institute of Technology (UK); CTC, Center of Technology of Meat (IRTA; Girona, Spain); FVM, Facultad de Veterinaria, Universidad Complutense (Madrid, Spain); HRM, Hospital Ramón y Cajal (Madrid, Spain); INRA, Institut National de la Recherche Agronomique (Jouy-en-Josas, France); IPLA, Instituto de Productos Lácteos de Asturias (Oviedo, Spain); Matforsk (Ås, Norway); NCDO, National Collection of Dairy Organisms (Reading, UK); TNO, Nutrition and Food Research (Zeist, The Netherlands); UV, Universidad de Valencia (Burjasot, Spain); UG, Universidad de Granada (Granada, Spain).

^c Sensitivity to cell free supernatant of *E. faecalis* C901, assayed by the spot-on-lawn method. Sensitivity scale: + (<9mm), ++ (10-11 mm), +++ (11-12mm), and ++++ (12-14 mm), reflect the degree of sensitivity according to the diameter of the inhibition halo showed in brackets; -, resistant.

Table 3. Minimal inhibition concentrations (MICs) of different antibiotics for *E. faecalis* C901.

Antibiotic ^a	MICs ($\mu\text{g ml}^{-1}$)	S/R ^b
AUG	1/0.5	4/8
AMP	2	$\leq 8/\geq 16$
CHL	16	$\leq 8/\geq 32$
CIP	2	$\leq 1/\geq 4$
CLI	>2	
ERY	2	$\leq 0.5/\geq 8$
FOS	32	$\leq 64/\geq 256$
GEN	128	$\leq 500/>500$
IMI	2	
MUP	256	
NIT	64	
LNZ	2	$\leq 2/\geq 8$
OXA	>2	
PEN	4	
Q/D	>4	$\leq 1/\geq 4$
RIF	≤ 1	$\leq 1/\geq 4$
STR	≤ 1000	$\leq 1000/\geq 1000$
TEI	$\leq 0,5$	$\leq 8/\geq 32$
SXT	$\leq 1/38$	
TET	>8	$\leq 4/\geq 16$
VAN	4	$\leq 4/\geq 32$

^a Antibiotic abbreviations are described in Materials and Methods

^b NCCLS, 2002, (38) was used to determine sensitivity (S) or resistance (R)

Figure 1

A

B

Figure 2

Figure 3

Peptide	Amino acid sequence and alignment	MW(t)	MW(e)	Reference
EntC1 (1071A)	ESVFSKIGNAVGPAAYWILKGLGNMSDVNQADRI ¹ NRKKH	4,286	4,284	This study; (4, 5, 19)
LcnG α	GTWDDIGOGIGRVAYWVGKAMGNMSDVNQASRI ¹ NRKKKH	4,376	4,346	(42)
LcnQ α	SI G V KA	4,260	4,260	(55)
EntC2	GPGKWLPWLQPAYDFVAGLAKGIGKEGNKNKWKNV	3,869	3,867	This study
1071B	GPGKWLPWLQPAYDFVTGLAKGIGKEGNKNKWKNV	3,899	3,898	(4, 5, 19)
LcnG β	KKWGWLAWVDPAYEFIKGFGKGA ¹ IKEGNKDKWKNI	4,109	4,110	(42)
LcnQ β	E G L	4,018	4,018	(55)

Figure 4

Figure 5