Applied and Environmental Microbiology, July 2010, p. 4542–4545 Vol. 76, No. 13
0099-2240/10/$12.00 doi:10.1128/AEM.02264-09
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Enterocin X, a Novel Two-Peptide Bacteriocin from *Enterococcus faecium* KU-B5, Has an Antibacterial Spectrum Entirely Different from Those of Its Component Peptides▽

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Received 20 September 2009/Accepted 12 April 2010

Enterocin X, composed of two antibacterial peptides (Xα and Xβ), is a novel class IIb bacteriocin from *Enterococcus faecium* KU-B5. When combined, Xα and Xβ display variably enhanced or reduced antibacterial activity toward a panel of indicators compared to each peptide individually. In *E. faecium* strains that produce enterocins A and B, such as KU-B5, only one additional bacteriocin had previously been known.

Bacteriocins are gene-encoded antibacterial peptides and proteins. Because of their natural ability to preserve food, they are of particular interest to researchers in the food industry. Bacteriocins are grouped into three main classes according to their physical properties and compositions (11, 12). Of these, class IIb bacteriocins are thermostable non-lanthionine-containing two-peptide bacteriocins whose full antibacterial activity requires the interaction of two complementary peptides (8, 19). Therefore, two-peptide bacteriocins are considered to function together as one antibacterial entity (14).

Enterocins A and B, first discovered and identified about 12 years ago (2, 3), are frequently present in *Enterococcus faecium* strains from various sources (3, 5, 6, 9, 13, 16). So far, no other bacteriocins have been identified in these strains, except the enterocin P-like bacteriocin from *E. faecium* JCM 5804^T (18). Here, we describe the characterization and genetic identification of enterocin X in *E. faecium* KU-B5. Enterocin X (identified after the enterocin P-like bacteriocin was discovered) is a newly found class IIb bacteriocin in *E. faecium* strains that produce enterocins A and B.

Enterocins A and B in *Enterococcus faecium* KU-B5. *E. faecium* KU-B5, a thermotolerant lactic acid bacterium screened from sugar apples in Thailand, has the ability to multiply and express antibacterial activity across a wide range of temperatures (20 to 43°C). Bacteriocin activity was tested by the critical dilution spot-on-lawn method (21) and expressed in activity units (AU) per milliliter. The strongest antibacterial activity of *E. faecium* was observed when it was cultured at 37°C in de Man-Rogosa-Sharpe (MRS) broth, with a wide spectrum of targets, including *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Bacillus*, and *Listeria* (Table 1). Since enterocins A and B are frequently found in many antibacterial *E. faecium* strains, we initially examined the enterocin A- and B-encoding genes in *E. faecium* KU-B5 by using PCR with specific primers (5′-TGTACGAAGTGCATTCTCAA-3′ and 5′-TATTAAAGGACCGGGATCTA-3′ for enterocin A; 5′-ACTCTAAAAGGAGCGAGTTT-3′ and 5′-AGAGCTGGGGATGAAATATT-3′ for enterocin B). As expected, these genes were detected in genomic DNA of *E. faecium* KU-B5. However, the pooled activities from enterocins A and B could not explain the total activities in the culture supernatant of *E. faecium* KU-B5, such as activity toward *Bacillus circulans* JCM 2504^T (Table 1). On the other hand, the gene encoding the enterocin P-like bacteriocin (18) was not detected in *E. faecium* KU-B5. Thus, we assumed that other bacteriocins exist in *E. faecium* KU-B5, and we obtained the purified bacteriocins, as described below.

Isolation of antibacterial substances and identification of a novel two-peptide bacteriocin. The supernatant from an overnight culture (at 37°C in MRS broth without shaking) of *E. faecium* KU-B5 was collected and subjected to a three-step purification process (10), including cation-exchange chromatography (SP Sepharose Fast Flow column), hydrophobic interaction (Sep-Pak Plus cartridge), and reverse-phase high-performance liquid chromatography (HPLC; Resource RPC 3-ml column) in sequence. Four antimicrobial substances were separated; of these, the 4,830.5- and 5,463.8-Da substances were identified according to their sizes as enterocins A and B, respectively. The other two substances (4,420.1 Da and 4,068.5 Da; pI = 8.83 and 10.32, respectively) were 40- and 37-residue cationic peptides, respectively, without posttranslationally modified bases and displayed synergistic antibacterial activity on the indicator lawn (Fig. 1). A database search for sequence similarity revealed no known bacteriocins; however, in their sequences, we detected the GXXXG motifs, which are suggested to be involved in helix-helix interaction (19) and often found in most two-peptide bacteriocins (14) (Fig. 1). The characteristics mentioned above fit typical features of two-peptide bacteriocins (17); therefore, they were named enterocin Xα

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▽ Published ahead of print on 23 April 2010.

TABLE 1. Antibacterial spectra of bacteriocins found in *Enterococcus faecium* KU-B5

Indicator strain	Antibacterial activity in culture supernatant (AU/ml)	MIC (nM)[a]				
		EntA	EntB	EntXα	EntXβ	EntXα+Xβ[b]
Enterococcus faecalis JCM 5803^T	1,600	64.7	86.9	50.8	813	203[e] (**0.5**; 8.0)
Enterococcus faecalis OU510	1,600	32.3	174	102	813	406[e] (**0.5**; 4.0)
Enterococcus faecium JCM 5804^T	200	NA[f]	NA	NA	NA	813[c] (**>16**; >16)
Enterococcus faecium TUA 1344L	3,200	129	43.4	50.8	813	50.8[e] (**2.0**; 32)
Enterococcus faecium KU-B5	100	NA	NA	NA	NA	1,630[c] (**>8.0**; >8.0)
Enterococcus hirae ATCC 10541	6,400	129	5.44	50.8	406	50.8[e] (**2.0**; 16)
Lactobacillus plantarum ATCC 14917^T	3,200	259	86.9	102	813	203[e] (**1.0**; 8.0)
Lactobacillus sakei subsp. *sakei* JCM 1157^T	12,800	16.2	5.44	50.8	102	203[e] (**0.5**; 1.0)
Lactococcus lactis subsp. *cremoris* ATCC 19257^T	6,400	4,140	NA	813	NA	12.7[c] (**130**; >1,020)
Staphylococcus aureus subsp. *aureus* ATCC 12600^T	NA	NA	NA	NA	NA	NA
Streptococcus salivarius JCM 5707^T	NA	NA	NA	NA	NA	NA
Bacillus cereus JCM 2152^T	400	NA	NA	NA	NA	NA
Bacillus circulans JCM 2504^T	6,400	NA	NA	NA	3,250	813[c] (>16; **8.0**)
Bacillus coagulans JCM 2257^T	200	8,280	89,000	NA	3,250	1,630[c] (>8.0; **4.0**)
Bacillus subtilis JCM 1465^T	400	4,140	44,500	NA	3,250	1,630[c] (>8.0; **4.0**)
Listeria innocua ATCC 33090^T	400	1,040	NA	25.4	3,250	406[d] (**0.13**; 16)
Micrococcus luteus NBRC 12708	NA	NA	NA	NA	NA	NA

[a] MICs of enterocin A (EntA), enterocin B (EntB), enterocin Xα (EntXα), enterocin Xβ (EntXβ), and the combination of enterocins Xα and Xβ (EntXα+Xβ), determined by the spot-on-lawn method (21).

[b] The MICs of EntXα+Xβ represent the antibacterial activities of the combination of equimolar amounts of enterocin Xα and enterocin Xβ against a panel of indicator bacteria. The values in parentheses represent two MIC ratios: EntXα/(1/2 × EntXα+Xβ) and EntXβ/(1/2 × EntXα+Xβ). The activity change of at least 4-fold is considered significant. For EntXα and EntXβ, the enterocin with higher activity against a particular bacterium (i.e., lower MIC) is used to evaluate the extent in activity change (numbers in boldface type in parentheses) after combination.

[c] The activity of EntXα+Xβ was enhanced compared with that of EntXα or EntXβ.

[d] The activity of EntXα+Xβ was reduced compared with that of EntXα or EntXβ.

[e] The activity of EntXα+Xβ was not significantly changed compared with that of EntXα or EntXβ.

[f] NA, no activity detected at the highest concentration of applied EntA (132,500 nM), EntB (89,000 nM), EntXα (6,500 nM), EntXβ (6,500 nM), or EntXα+Xβ (6,500 nM).

(4,420.1 Da) and enterocin Xβ (4,068.5 Da), together comprising enterocin X.

Individual enterocins Xα and Xβ exhibited narrow-spectrum weak-to-moderate antibacterial activities, similar to those of plantaricin E/F and plantaricin J/K (1), but enterocin Xα had stronger antilisterial activity (Table 1). Synergistic antibacterial activity was observed when enterocins Xα and Xβ were assessed on the indicator lawn (Fig. 1). However, when they were mixed beforehand in equimolar amounts, the combined antibacterial activity was not uniformly enhanced toward a panel of indicator bacteria, with activity 0.13- to 130-fold or 1.0- to 1,020-fold that of enterocin Xα or enterocin Xβ alone, respectively (Table 1). Therefore, depending on indicator strains, the combined antibacterial activity was either enhanced (greatly or slightly) or reduced. Thus, rather than saying that enterocins Xα and Xβ function together as one antibacterial entity (14), it is better to say that enterocins Xα and Xβ presenting together become "a new bacteriocin" with an entirely different antibacterial spectrum than those of their component peptides.

Genetic region encoding enterocin X. The structural gene of enterocin Xα (*enxA*) was discovered using PCR with degenerate primers corresponding to Trp^6-Gly^{14} (forward) and Leu^{35}-His^{27} (reverse). The neighboring DNA regions were extended by means of single-specific-primer PCR (20) and inverse PCR (15, 16). In the acquired 2,047-bp consecutive DNA fragment that contains the genes for enterocins Xα (*enxA*) and Xβ (*enxB*), six open reading frames (ORFs) were sequentially recognized according to the rules described by Harley and Reynolds (7) (Fig. 2). The functions of the *orfX1*, *orfX2*, and *orfX3* genes are unknown. The *orfX4* gene, located downstream of *enxB*, may be the immunity gene for enterocin X, based on its location and predicted product, a 73-residue, hydrophobic, and cationic (pI = 10.2) protein with two putative transmembrane domains (4, 12).

The *enxB* gene is located 19 nucleotides downstream from *enxA* in the same direction, with no obvious *rho*-independent transcriptional terminator (invert repeats) between them. Thus, *enxA* and *enxB* may belong to the same transcriptional unit, an arrangement typical of genes encoding two-peptide bacteriocins. Both *enxA* and *enxB* encode a double-glycine-type prepeptide with an 18-residue leader sequence, representing the typical structure of class II bacteriocin prepeptides. Thus, enterocin X, composed of enterocins Xα and Xβ, was confirmed to be a novel two-peptide class II bacteriocin.

Interestingly, the upstream region of the enterocin X locus (from *orfX1* to the 5′ end of *enxA*; ~1 kb) is sequence similar with the upstream region of the enterocin B locus (Fig. 2). These sequence-similar regions contribute to the formation of almost-identical leader sequences on enterocin B and enterocin Xα prepeptides. Thus, enterocin Xα and enterocin B were suggested to have the same transport apparatus, maybe the same as that for enterocin A (6). Furthermore, the gene encoding enterocin X was also detected by PCR in other *E. faecium* strains that produce enterocins A and B (strains JCM 5804^T and WHE 81) (data not shown). These sequence-similar DNA regions, therefore, may imply the occurrence of gene rearrangement in bacteriocin genes.

In conclusion, this study not only reports the discovery of a novel two-peptide bacteriocin in *E. faecium* KU-B5 but also is the first to report the nonuniform change in antibacterial ac-

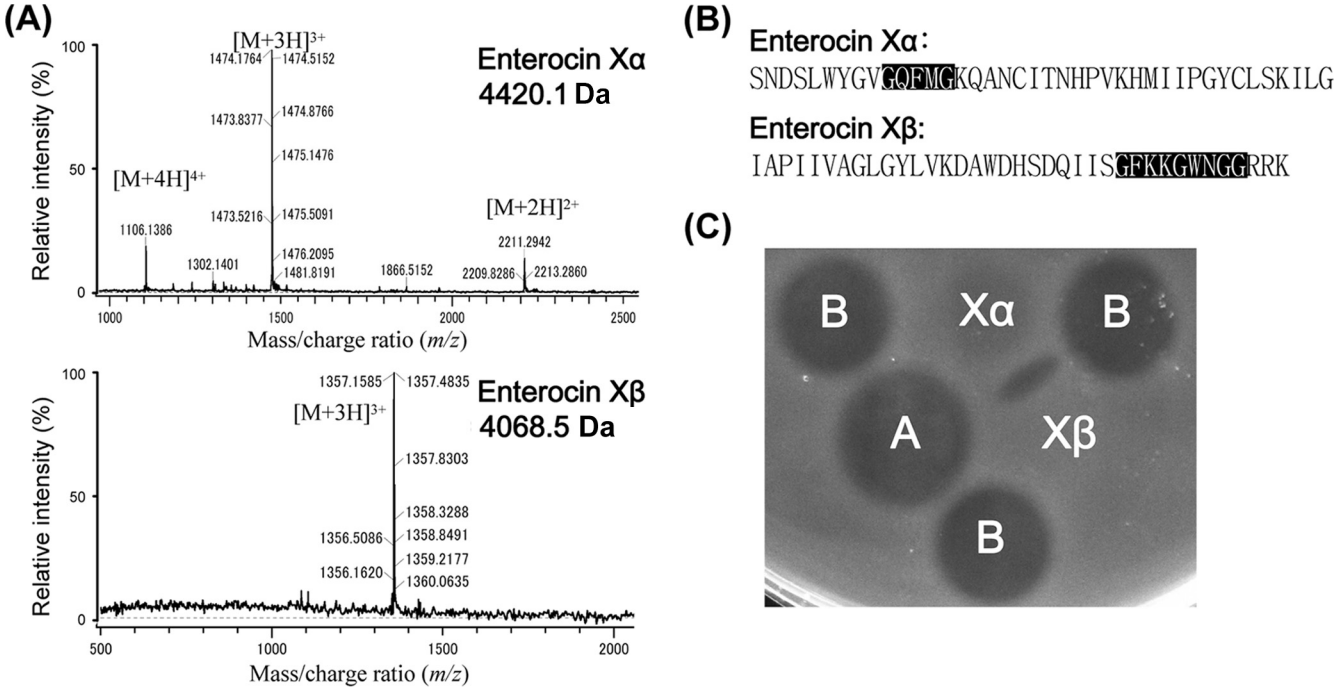

FIG. 1. Characteristics of enterocin Xα and enterocin Xβ. (A) Electrospray ionization tandem mass spectrometry spectra of purified enterocins Xα and Xβ. The observed molecular sizes are indicated in each panel. (B) Amino acid sequences of enterocins Xα and Xβ from N to C termini. The GXXXG motifs are shaded in black. (C) Synergism assay for pairs of enterocins A, B, Xα, and Xβ. Enterocins with the appropriate dilution were spotted onto the indicator lawn of *Enterococcus faecium* TUA 1344L. The additional inhibition zone that appears between enterocins Xα and Xβ depicts the synergic antibacterial activity.

tivity toward a panel of bacteria when two antibacterial peptides were combined. We also reported the interesting fact about the sequence-similar regions upstream of the enterocin X and B loci. In addition, the thermotolerant property of *E. faecium* KU-B5, the enterocin X producer, is beneficial in bacteriocin applications, such as silage production.

Nucleotide sequence accession numbers. The DNA sequences of the loci for enterocins A, B, and X in *E. faecium*

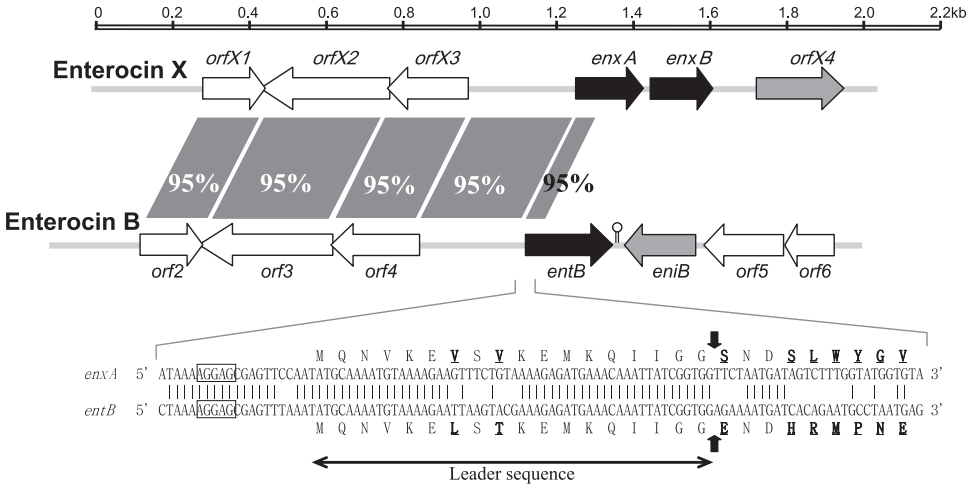

FIG. 2. Alignment of the DNA sequences containing the genetic determinants for enterocin X and enterocin B. The DNA sequence of the enterocin B locus is obtained from the *entB* locus in *Enterococcus faecium* BFE 900 (accession no. AF076604). The horizontal arrows denote ORFs, the black arrows represent the genes encoding a prepeptide with a putative double-glycine-type leader sequence, the gray arrows represent (putative) immunity genes, and the white arrows represent the genes with unknown functions. The *enxA* and *enxB* genes are the structural genes for enterocins Xα and Xβ, respectively. The *entB* and *eniB* genes are the structural gene and the immunity gene for enterocin B, respectively. The lollipop represents a stem-loop structure functioning as a *rho*-independent terminator. Upstream regions of enterocin X and B loci share a sequence-similar region (longer than 1 kb; >95% identity, as shown by the gray zones between their gene clusters). The 5′ ends of *enxA* and *entB*, as well as N-terminal parts of their translation products, show high sequence similarity. The identical nucleotide bases are denoted with a bar, and the diverse amino acid residues are indicated by underlined boldface type. The rectangles indicate the proposed ribosome-binding sites. The vertical black arrows indicate the putative cleavage sites for the prepeptides translated from *enxA* and *entB*.

KU-B5 have been deposited in the DDBJ/EMBL/GenBank databases. The accession numbers are AB292463, AB292464, and AB430879, respectively.

We thank Japan Educational Exchanges and Services for providing the JINNAI international student scholarship as financial support to C.-B.H. This work was partially supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS) and the JSPS-NRCT Core University Program on "Development of Thermotolerant Microbial Resources and Their Applications."

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