

Development of Bacteriocinogenic Strains of *Saccharomyces cerevisiae* Heterologously Expressing and Secreting the Leaderless Enterocin L50 Peptides L50A and L50B from *Enterococcus faecium* L50[∇]

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A segregationally stable expression and secretion vector for *Saccharomyces cerevisiae*, named pYABD01, was constructed by cloning the yeast gene region encoding the mating pheromone α -factor 1 secretion signal ($MF\alpha I_s$) into the *S. cerevisiae* high-copy-number expression vector pYES2. The structural genes of the two leaderless peptides of enterocin L50 (EntL50A and EntL50B) from *Enterococcus faecium* L50 were cloned, separately (*entL50A* or *entL50B*) and together (*entL50AB*), into pYABD01 under the control of the galactose-inducible promoter P_{GAL1} . The generation of recombinant *S. cerevisiae* strains heterologously expressing and secreting biologically active EntL50A and EntL50B demonstrates the suitability of the $MF\alpha I_s$ -containing vector pYABD01 to direct processing and secretion of these antimicrobial peptides through the *S. cerevisiae* Sec system.

Lactic acid bacteria (LAB) are widely known for their ability to produce a variety of ribosomally synthesized proteins or peptides, referred to as bacteriocins, displaying antimicrobial activity against a broad range of gram-positive bacteria and, to a lesser extent, gram-negative bacteria, including spoilage and food-borne pathogenic microorganisms (11, 19, 33, 34, 36, 37). These antimicrobials may be classified into three main classes: (i) the lantibiotics, or posttranslationally modified peptides; (ii) the nonmodified, small, heat-stable peptides; and (iii) the large, heat-labile protein bacteriocins. Class II bacteriocins are further grouped into five subclasses: the subclass IIa (pediocin-like bacteriocins containing the N-terminal conserved motif YGNGVxC), the subclass IIb (two-peptide bacteriocins), the subclass IIc (leaderless bacteriocins), the subclass II d (circular bacteriocins), and the subclass II e (other peptide bacteriocins) (17, 19, 21, 37). All lantibiotics and most class II bacteriocins are synthesized as biologically inactive precursors containing an N-terminal extension (the so-called double-glycine-type leader sequence or the Sec-dependent signal peptide), which is cleaved off concomitantly with externalization of biologically active bacteriocins by a dedicated ATP-binding cassette transporter and its accessory protein or by the Sec system and the signal peptidases, respectively (11, 17). Interestingly, only a few bacteriocins described to date are synthesized without an N-terminal extension, including enterocin L50 (L50A and L50B) (8), enterocin Q (EntQ) (10), enterocin EJ97 (41), and the bacteriocin LsbB (20).

In recent years, there has been an increasing interest in the

application of bacteriocinogenic microorganisms and/or their bacteriocins as biopreservatives to guarantee the safety and quality of foods and beverages, such as fermented vegetables and meats, dairy and fish products, and wine and beer (12, 15, 16, 39, 47). Three main strategies for the use of bacteriocins as food biopreservatives have been proposed: (i) addition of a purified/semipurified bacteriocin preparation as a food additive; (ii) use of a substrate previously fermented by a bacteriocin-producing strain as a food ingredient; and/or (iii) inoculation of a culture to produce the bacteriocin in situ in fermented foods (13, 15). The lantibiotic nisin A is the most widely characterized bacteriocin and the only one that has been legally approved in more than 48 countries as a food additive for use in certain types of cheeses (13, 16). Likewise, nisin A has been approved as a beer additive in Australia and New Zealand (16). However, the difficulties encountered in addressing the regulatory approval of new bacteriocins as food additives have spurred the development of the other bacteriocin-based food biopreservation strategies (13, 17).

Beer is a beverage with a remarkable microbiological stability and is considered as a food substrate difficult to spoil. However, some LAB, such as *Lactobacillus brevis*, *Lactobacillus lindneri*, and *Pediococcus damnosus*, are able to spoil beer and are recognized as the most hazardous bacteria for breweries, being responsible for approximately 70% of microbial beer spoilage incidents (40, 47). The ever-growing consumer demand for less-processed and less chemically preserved foods and beverages is promoting the development of alternative biocontrol strategies, such as those based on the use of bacteriocins as biopreservatives (12, 15, 39, 47). However, beyond the strict requirements to fulfill legal regulations, the commercial application of bacteriocins as beer additives is hindered mainly by low bacteriocin production yields and increases in production costs (44). Considering that *Saccharomyces cerevisiae* is commonly used as starter culture for brewing (24, 28,

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TABLE 1. Microorganisms and plasmids used in this study

Strain or plasmid	Relevant characteristics ^a	Source or reference ^b
Bacterial strains		
<i>E. faecium</i> L50	EntL50 (EntL50A and EntL50B), EntP, and EntQ producer	DNBTA
<i>P. damnosus</i> 4797	Indicator microorganism, EntL50 ^s -EntP ^s -EntQ ^r	CECT
<i>E. coli</i> TOP10	Host strain, F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>recA1</i> <i>deoR</i> <i>araD139</i> Δ(<i>ara-leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (Str ^r) <i>endA1</i> <i>nupG</i>	Invitrogen
<i>E. coli</i> MAX Efficiency DH5α	Host strain, <i>supE44</i> Δ <i>lacU169</i> (φ80 <i>lacZ</i> Δ <i>M15</i>) <i>hsdR17</i> <i>recA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Invitrogen
Yeast (<i>S. cerevisiae</i>) strains		
INVSc1	Host strain, MATα <i>his3Δ1</i> <i>leu2</i> <i>trp1-289</i> <i>ura3-52</i> ; His ⁻ Leu ⁻ Trp ⁻ Ura ⁻	Invitrogen
INVSc1-α	<i>S. cerevisiae</i> INVSc1 derivative carrying pYABD01, Amp ^r	This work
L50A-20	<i>S. cerevisiae</i> INVSc1 derivative carrying pYABD02, EntL50A producer, Amp ^r	This work
L50B-4	<i>S. cerevisiae</i> INVSc1 derivative carrying pYABD03, EntL50B producer, Amp ^r	This work
L50AB-2	<i>S. cerevisiae</i> INVSc1 derivative carrying pYABD04, EntL50A producer, Amp ^r	This work
Plasmids		
PCR2.1-TOPO	3.9-kb cloning vector, Amp ^r Kan ^r	Invitrogen
pPICZαA	<i>P. pastoris</i> 3.6-kb protein expression and secretion vector carrying a methanol-inducible promoter (P _{AOX1}), 5'AOX1 region, MFα1 _s , Zeo ^r	Invitrogen
pYES2	<i>S. cerevisiae</i> 5.9-kb protein expression vector carrying galactose-inducible promoter P _{GAL1} , Ura3, Amp ^r	Invitrogen
pRSETB- <i>entL50A</i>	pRSETB derivative carrying <i>entL50A</i>	8
pRSETB- <i>entL50B</i>	pRSETB derivative carrying <i>entL50B</i>	8
pTBS01	PCR2.1-TOPO derivative carrying MFα1 _s	This work
pTBS02	PCR2.1-TOPO derivative carrying <i>entL50A</i>	This work
pTBS03	PCR2.1-TOPO derivative carrying <i>entL50B</i>	This work
pYABD01	pYES2-derived expression and secretion vector carrying MFα1 _s	This work
pYABD02	pYABD01 derivative carrying <i>entL50A</i>	This work
pYABD03	pYABD01 derivative carrying <i>entL50B</i>	This work
pYABD04	pYABD02 derivative carrying MFα1 _s fused in frame to <i>entL50B</i>	This work

^a MFα1_s, yeast gene region encoding the mating pheromone α-factor 1 secretion signal (MFα1_s).

^b Abbreviations: CECT, Colección Española de Cultivos Tipo (Valencia, Spain); DNBTA, Departamento de Nutrición, Bromatología y Tecnología de los Alimentos, Facultad de Veterinaria, Universidad Complutense de Madrid (Madrid, Spain).

35), a novel beer biopreservation strategy based on the development of bactericidal *S. cerevisiae* brewing strains has been proposed to overcome the aforementioned challenges (44, 46, 47). In this respect, the heterologous production of LAB bacteriocins, namely, pediocin PA-1 (PedPA-1) from *Pediococcus acidilactici* PAC1.0 and plantaricin 423 from *Lactobacillus plantarum* 423, by laboratory strains of *S. cerevisiae* has been reported (44, 46).

Enterocin L50 (EntL50) is a commonly found bacteriocin composed of two highly related leaderless antimicrobial peptides, enterocin L50A (EntL50A) and enterocin L50B (EntL50B), which possesses a broad antimicrobial spectrum against LAB, food-borne pathogenic bacteria, and human and animal clinical pathogens (8, 9, 10, 11). Previous work by our group showed that EntL50 (EntL50A and EntL50B) may be used as a beer biopreservative to inhibit the growth of beer spoilage bacteria (1). Therefore, genetically engineered strains of *S. cerevisiae* heterologously expressing and secreting EntL50A and EntL50B have been developed in this work. For this purpose, we constructed the segregationally stable expression and secretion vector pYABD01, which allowed the secretion of biologically active EntL50A and EntL50B directed by MFα1_s through the *S. cerevisiae* Sec system.

MATERIALS AND METHODS

Microorganisms, plasmids, media, and culture conditions. The sources and relevant genotypes of the microorganisms and plasmids used in this work are listed in Table 1. The EntL50 (EntL50A and EntL50B)-producing strain *Enterococcus faecium* L50 (8) and the indicator strain *P. damnosus* CECT4797 (EntL50 sensitive; EntL50^s) (1) were grown aerobically in MRS broth (pH 6.2; Oxoid Ltd., Basingstoke, United Kingdom) at 30°C. *Escherichia coli* cells (Invitrogen Life Technologies, Carlsbad, CA) were propagated in Luria-Bertani (LB) broth (Sigma-Aldrich Inc., St. Louis, MO) at 37°C with shaking (200 to 250 rpm). Kanamycin (50 μg/ml), ampicillin (Amp) (50 to 100 μg/ml), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (80 μg/ml) (Sigma-Aldrich) were added to LB medium for selection of *E. coli* transformants. *S. cerevisiae* INVSc1 (Invitrogen) was cultured in YPD medium (10 g/liter yeast extract [Oxoid], 20 g/liter peptone [Oxoid], and 20 g/liter glucose [Panreac Química S. A., Barcelona, Spain]) (44) at 30°C with shaking (200 to 250 rpm). Yeast transformants were grown in YPD medium supplemented with Amp (100 or 50 μg/ml in liquid or solid medium, respectively) and/or in synthetic complete minimal medium (SC) containing 0.67% (wt/vol) yeast nitrogen base (Invitrogen) without amino acids, 2% (wt/vol) glucose, and all the required growth factors except uracil (SC^{-Ura}) (44). Solid media contained 1.5 or 2% (wt/vol) agar (Oxoid) for LAB and *E. coli* or *S. cerevisiae*, respectively. Soft MRS agar used in the spot-on-agar test (SPAT) described below contained 0.8% (wt/vol) agar.

DNA isolation and manipulation. Small- and large-scale plasmid DNA isolation from *E. coli* recombinants was carried out using the High Pure plasmid isolation kit (Roche Diagnostics SL, Madrid, Spain) and the QIAGEN Plasmid Midi kit (Qiagen GmbH, Hilden, Germany), respectively. Total genomic DNA from recombinant yeasts was isolated using the Wizard DNA Purification kit (Promega Corporation, Madison, WI). Oligonucleotide primers (Table 2) were obtained from Sigma-Genosys Ltd. (Cambridge, United Kingdom). For PCR amplifications, samples were subjected to an initial cycle of denaturation (97°C for 2 min), followed by 35 cycles of denaturation (94°C for 45 s), annealing (50 to 56°C for 30 s), and elongation (72°C for 30 to 60 s), ending with a final extension step at 72°C for 7 min. The PCR-generated fragments were extracted from 0.8% (wt/vol) agarose gels using the Real Clean Matrix kit (Durviz SLU, Madrid, Spain) and purified using the QIAquick PCR purification kit (Qiagen). Nucleotide sequencing of both strands of purified PCR products was done at the DNA Sequencing Service of Sistemas Genómicos (Valencia, Spain). Platinum

TABLE 2. Primers and PCR products used in this study

Primer or PCR product	Nucleotide sequence (5'–3') ^a or PCR product description ^b	Fragment(s) amplified
Primers		
Alfa1-HindIII	<u>AAGCTT</u> ACGATGAGATTTTCCTTCAATTTTTACTGC	α1
Alfa3-XbaI	TCTAGAAAGCTGGCGGCCCGCGCTC	α1
Alfa4-SmaI	<u>CCCGGG</u> ACGATGAGATTTTCCTTCAATTTTTACTGC	α-L50B
L50A1-XhoI	<u>CTCGAGAAA</u> AAGTGGGAGCAATCGCAAAATTAGTAGCAAAG	L50A
L50A2-XbaI	<u>TCTAGAGTATA</u> ACCCTCCGGGATTTTAAATATGTTTTTAAATCCACTCAATG	L50A
L50B1-XhoI	<u>CTCGAGAAA</u> AAGTGGGAGCAATCGCAAAACTAGTGAC	L50B
L50B2-XbaI	<u>TCTAGAAA</u> ACATTAATGCTTTTTAGCCATTTTCAATTTTGATC	L50B, α-L50B
pYES2-F	AACCCCGGATCGGACTACTAG	α2
pYES2-R	GGCGTGAATGTAAGCGTGAC	α2
PCR products		
Fragment α1	345-bp HindIII-XbaI fragment containing the Kozak sequence fused to <i>MFα1_s</i>	
Fragment α2	442-bp fragment containing the Kozak sequence fused to <i>MFα1_s</i>	
Fragment L50A	170-bp XhoI-XbaI fragment containing <i>MFα1_s</i> fused to <i>entL50A</i>	
Fragment L50B	154-bp XhoI-XbaI fragment containing <i>MFα1_s</i> fused to <i>entL50B</i>	
Fragment α-L50A	422-bp HindIII-XbaI fragment containing <i>MFα1_s</i> fused to <i>entL50A</i>	
Fragment α-L50B	406-bp SmaI-XbaI fragment containing <i>MFα1_s</i> fused to <i>entL50B</i>	

^a Cleavage sites for restriction enzymes are underlined; Kozak sequence (ACGATGA), and the nucleotides encoding the Kex2 signal cleavage site (AAAAGA) of the mating pheromone α-factor 1 secretion signal (*MFα1_s*) are shown in bold.

^b *MFα1_s* refers to the yeast gene region encoding *MFα1_s*, including the Kex2 cleavage site.

Taq polymerase (Invitrogen), DNA restriction enzymes (New England Biolabs Inc., Beverly, MA), and T4 DNA ligase (Promega) were used as suggested by the manufacturers. *E. coli* TOP10 and *E. coli* MAX Efficiency DH5α competent cells, as well as *S. cerevisiae* INVSc1 competent cells from the S.c. EasyCom transformation kit, were transformed according to the supplier's instructions (Invitrogen).

Construction of protein expression and secretion vector pYABD01 for *S. cerevisiae*. The strategy employed for construction of pYABD01 is depicted in Fig. 1. The *Pichia pastoris* expression and secretion vector pPICZαA was used as the template for PCR amplification of fragment α1 containing a Kozak translation initiation sequence (ACGATGA) in addition to the gene region encoding the mating pheromone α-factor 1 secretion signal (*MFα1_s*), which includes the Kex2 signal cleavage site (Glu-Lys-Arg) required for processing of the fusion protein. The purified fragment α1 was cloned into PCR2.1-TOPO (Invitrogen), generating the plasmid pTBS01, which was chemically transformed into *E. coli* TOP10 cells. Plasmid pTBS01 was digested with HindIII and XbaI and the resulting 339-bp fragment purified from the agarose gel and ligated with T4 DNA ligase into the HindIII and XbaI sites of the *S. cerevisiae* protein expression vector pYES2 to give plasmid pYABD01, in which fragment α1 is under the control of the P_{GAL1} promoter (induced by galactose and repressed by glucose) and the corresponding enhancer sequences. The recombinant plasmid was chemically transformed into *E. coli* MAX Efficiency DH5α cells and transformants selected on LB plates with Amp (50 μg/ml) at 37°C for 24 h. The plasmid pYES2 was also chemically transformed into this host, and the resulting strain was used as a control. The presence of the plasmid pYABD01 in transformed cells was confirmed by PCR and plasmid isolation and restriction analysis, and the correct nucleotide sequence of pYABD01 was confirmed by DNA sequencing as described above. Primers used for PCR amplification and DNA sequencing were designed from the nucleotide sequence of plasmids pPICZαA and pYES2 (Invitrogen website) (Table 2).

In order to assess the segregational stability of pYABD01 (carrying *Ura3*), this plasmid was transformed into *S. cerevisiae* INVSc1 (auxotrophic for Ura) competent cells, and the resulting *S. cerevisiae* INVSc1-α(pYABD01) transformant (see below) was grown in SC^{-Ura} overnight (optical density at 600 nm [OD₆₀₀] of 2.0). Subsequently, the culture was transferred to YPD broth (initial inoculum of approximately 1 × 10⁵ CFU/ml) and further incubated for 50 generations (seven serial culture transfers using 0.5% inocula every 24 h) to determine the plasmid stability without selective pressure during continuous exponential growth phase. At 24-h intervals, samples were withdrawn from the culture for bacterial counting on SC^{-Ura} and YPD plates.

Cloning of *EntL50A* (*entL50A*) and *EntL50B* (*entL50B*) structural genes, separately and together, in pYABD01. The strategy employed for cloning of *entL50A* and *entL50B* in pYABD01 is summarized in Fig. 1. The recombinant plasmids pRSETB-*entL50A* and pRSETB-*entL50B*, previously constructed (8), were used as DNA templates for PCR amplification of fragments L50A and L50B carrying the nucleotide sequence encoding the Kex2 signal cleavage site (AAAAGA) of

MFα1_s fused to *entL50A* and *entL50B*, respectively. The purified fragments L50A and L50B were cloned into the plasmid pYABD01, essentially as described above, generating the recombinant plasmids pYABD02 and pYABD03, respectively. In these plasmids, *entL50A* and *entL50B* are fused in frame to *MFα1* and gene expression is under the control of P_{GAL1}. The recombinant plasmids were chemically transformed into *E. coli* MAX Efficiency DH5α cells and *S. cerevisiae* INVSc1 cells, and the resulting *S. cerevisiae* L50A and *S. cerevisiae* L50B transformants were selected on SC^{-Ura} plates at 30°C for 2 to 4 days. In order to express and secrete EntL50A and EntL50B simultaneously, the recombinant plasmid pYABD04 was constructed and analyzed essentially as described for pYABD02 and pYABD03 (Fig. 1). Briefly, the fragment α-L50B, which contains a Kozak sequence and *MFα1* fused to *entL50B*, was cloned into pYABD02 downstream of the fragment α-L50A, generating the plasmid pYABD04. This plasmid was transformed into *S. cerevisiae* INVSc1 competent cells, generating *S. cerevisiae* L50AB transformants. The plasmid pYABD01, lacking the bacteriocin structural genes, was also transformed into *S. cerevisiae* INVSc1, and the resulting *S. cerevisiae* INVSc1-α strain was used as a control.

The presence of pYABD01 in *S. cerevisiae* INVSc1-α, pYABD02 in *S. cerevisiae* L50A, pYABD03 in *S. cerevisiae* L50B, and pYABD04 in *S. cerevisiae* L50AB transformants was confirmed by PCR and plasmid isolation and restriction analysis, and the correct nucleotide sequences of the recombinant plasmids were confirmed by DNA sequencing as described above. Primers used for PCR amplification and DNA sequencing were designed from the published nucleotide sequence of the *EntL50* (*entL50A* and *entL50B*) operon (8) and plasmid pPICZαA (Table 2).

Detection and quantification of *EntL50A* and *EntL50B* heterologous production by antimicrobial and immunochemical assays. The direct antimicrobial activities of cultures from several *S. cerevisiae* L50A(pYABD02) and *S. cerevisiae* L50B(pYABD03) transformants were screened by using a SPAT essentially as described previously (7, 44). Briefly, transformants were precultured in both YPD and SC^{-Ura} broth overnight (OD₆₀₀ of 2.0). Subsequently, equal amounts (20 μl) of yeast precultures were spotted onto plates of both media supplemented with 20 g/liter galactose (Merck Farma y Química S. A., Barcelona, Spain) and 10 g/liter raffinose (Sigma-Aldrich) (YPGR and SCGR, respectively), and incubated at 30°C for 30 days. Periodically, one of the plates was overlaid with MRS soft agar previously seeded with approximately 1 × 10⁵ CFU/ml of the indicator microorganism *P. damnosus* CECT4797 and further incubated for 16 h. After incubation, the direct antimicrobial activity was detected by the presence of growth inhibition zones of the indicator microorganism around the spotted producer strain. Likewise, the direct antimicrobial activities of cultures from several *S. cerevisiae* L50AB(pYABD04) transformants were screened as described above but only on SCGR plates.

In order to determine bacteriocin production kinetics, selected *S. cerevisiae* L50A(pYABD02) and *S. cerevisiae* L50B(pYABD03) transformants, as well as *S. cerevisiae* L50AB(pYABD04) transformants, were precultured in YPD and

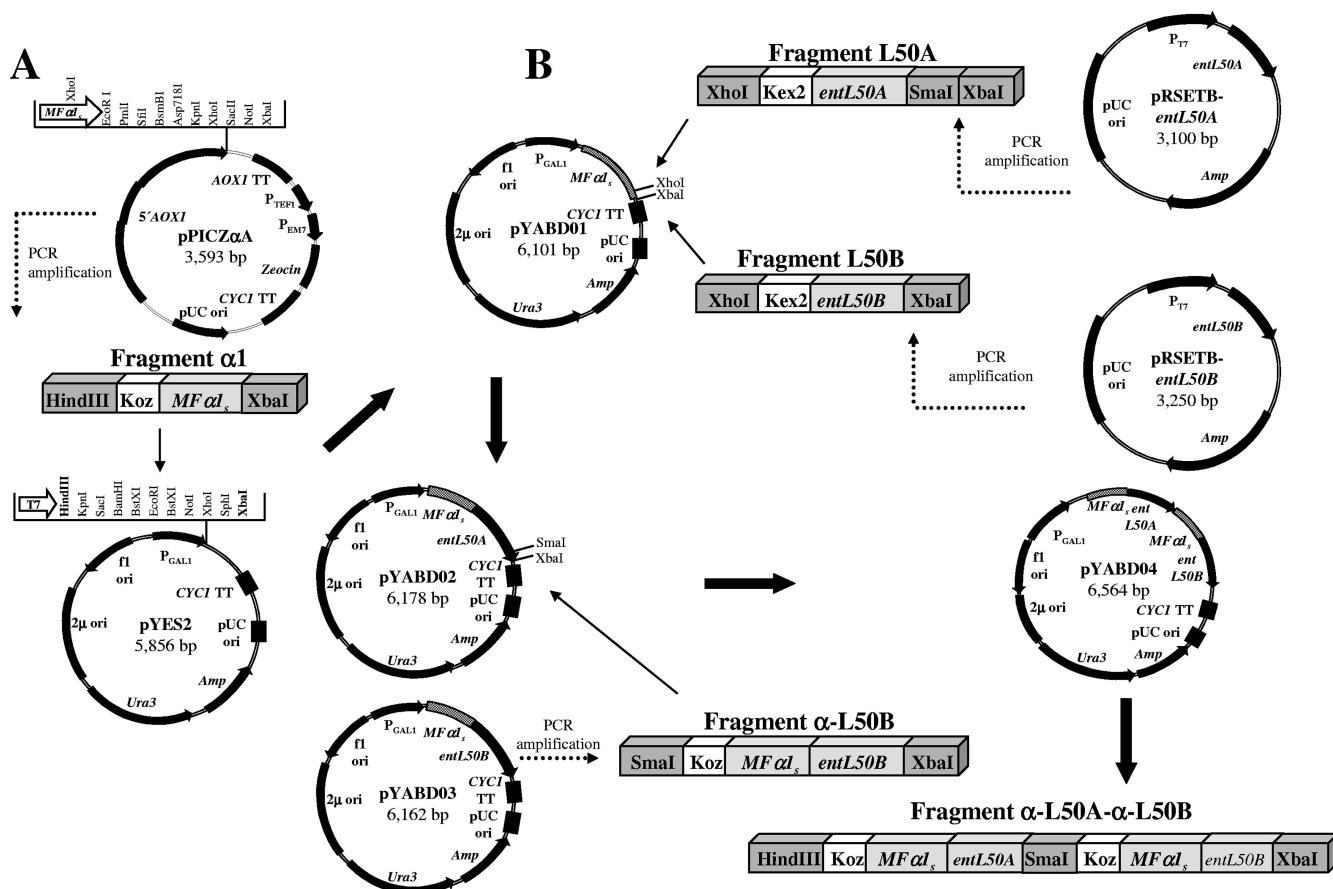


FIG. 1. (A) Construction of the *S. cerevisiae* protein expression and secretion vector pYABD01, derived from pPICZ α A and pYES2, containing the yeast gene region encoding the mating pheromone α -factor 1 secretion signal ($MF\alpha 1_s$), including the nucleotides encoding the Kex2 signal cleavage site under the control of the galactose-inducible promoter P_{GAL1} . (B) Construction of the recombinant plasmids pYABD02, pYABD03, and pYABD04, derived from pYABD01, containing $MF\alpha 1_s$ fused in frame to *entL50A* and/or *entL50B*. Plasmid sizes are given in base pairs. Only relevant restriction enzyme sites are shown. 5'*AOX1*, promoter region; *AOX1* TT, transcription termination; P_{TEF1} , transcription elongation factor 1, driving expression of the *Sh ble* gene in *Pichia*; P_{EM7} , constitutive promoter driving expression of the *Sh ble* gene in *E. coli*; *Zeocin* gene, zeocin resistance (*Sh ble* gene); *CYC1* TT, transcription terminator; pUC ori, maintenance and high-copy-number replication in *E. coli*; *Amp*, ampicillin resistance gene; *Ura3*, gene for selection of yeast transformants in uracil-deficient medium; 2 μ ori, maintenance and high-copy-number replication in yeast; f1 ori, rescue of single-stranded DNA; *entL50A*, structural gene of EntL50A; *entL50B*, structural gene of EntL50B; P_{T7} , T7 promoter driving expression of heterologous gene expression; Koz, Kozak translation initiation sequence of yeasts.

SC-Ura broth or in SC-Ura broth, respectively. Cells were harvested by centrifugation ($5,000 \times g$ at 4°C for 10 min), washed with YPD or SC-Ura (both without glucose), and resuspended to an OD_{600} of 2.0 in YPGR and SCGR broth. These recombinant yeasts were grown in YPGR and SCGR broth or in SCGR broth at 30°C for 8 days. Yeast growth (OD_{600}), cell dry weight (CDW), and the bacteriocin activity and concentration were determined periodically in duplicate. Supernatants were obtained by centrifugation ($12,000 \times g$ at 4°C for 10 min), further adjusted to pH 6.2, and filter sterilized through 0.22- μm -pore-size filters (Millipore Corp., Bedford, MA). Subsequently, aliquots of the supernatants were lyophilized and further dissolved to 1/20 of their original volume in 20 mM phosphate buffer (pH 6.0). For determination and quantification of bacteriocin activity, supernatants and 20-fold-concentrated supernatants (CS) were analyzed by an agar well diffusion test (ADT) (7) and a microtiter plate assay (MPA) (10, 25), respectively, using *P. damnosus* CECT4797 as the indicator microorganism. One bacteriocin unit (BU) was defined as the highest dilution of the supernatant causing 50% growth inhibition of the indicator microorganism. Cultures and supernatants from the *S. cerevisiae* INVSc1- α (pYABD01) transformants, lacking the bacteriocin structural genes, were used as negative controls in the antimicrobial assays.

For quantification of EntL50A and EntL50B heterologous production in the supernatants of the recombinant yeasts, a noncompetitive indirect enzyme-linked immunosorbent assay (NCI-ELISA) using rabbit polyclonal antibodies with specificity for EntL50A (anti-LR1-KLH) and EntL50B (anti-LR2-KLH) (14) was performed essentially as described previously (14, 22). The ELISA plates in-

cluded control wells coated with (i) 0.1 M sodium carbonate-bicarbonate buffer (pH 9.6) (coating buffer [CB])-MRS, CB-YPGR, and CB-SCGR broth to set the plate background level and (ii) six twofold dilutions of samples containing known concentrations of pure EntL50A and EntL50B (determined from the A_{280} using the molar extinction coefficient) in CB-MRS, CB-YPGR, and CB-SCGR broth to set plate standard curves. Supernatants from *S. cerevisiae* INVSc1- α (pYABD01) were used as negative controls in the immunochemical assays.

To measure the synergistic activities of heterologously produced EntL50A and EntL50B, supernatants from the *S. cerevisiae* L50A-20(pYABD02) and *S. cerevisiae* L50B-4(pYABD03) strains were challenged against *P. damnosus* CECT4797, separately and in combination, to achieve a 1:1 bacteriocin peptide ratio, by a SPAT and an MPA.

Purification and mass spectrometry analysis of recombinant EntL50A and EntL50B. EntL50A and EntL50B, heterologously produced by *S. cerevisiae* L50A-20(pYABD02) and *S. cerevisiae* L50B-4(pYABD03), respectively, were purified essentially as described previously (7). Briefly, yeast precultures grown in SC-Ura broth at 30°C were used to inoculate 1-liter cultures that were grown in SCGR broth at 30°C with shaking until maximum EntL50A and EntL50B concentrations were achieved (ca. 144 and 120 h, respectively). After the cells were removed by centrifugation ($12,000 \times g$ at 4°C for 30 min), the supernatants were subjected to precipitation with ammonium sulfate (50% [wt/vol]; Merck) and subsequently desalted by gel filtration (PD-10 columns; GE Healthcare Life Sciences, Barcelona, Spain). The obtained fractions were further subjected to

TABLE 3. Production and antimicrobial activity of recombinant EntL50A and EntL50B from *S. cerevisiae* L50A-20(pYABD02) and *S. cerevisiae* L50B-4(pYABD03) cultures, respectively^a

Time (h)	<i>S. cerevisiae</i> L50A-20(pYABD02)								<i>S. cerevisiae</i> L50B-4(pYABD03)							
	OD ₆₀₀ ^b	EntL50A concn in ^c :		Antimicrobial activity as assessed by:				SAA ^f (BU/ng EntL50A)	OD ₆₀₀	EntL50B concn in ^c :		Antimicrobial activity as assessed by:				SAA ^f (BU/ng EntL50B)
		ng/ml	ng/mg CDW	ADT (mm) ^d		MPA in ^e :				ng/ml	ng/mg CDW	ADT (mm) ^d		MPA in ^e :		
				S	CS	BU/ml	BU/mg CDW					S	CS	BU/ml	BU/mg CDW	
0	2.0	ND ^d	ND	ND	ND	ND	ND	NE	2.0	ND	ND	ND	ND	ND	ND	NE
2	2.0	0.7	0.4	ND	ND	ND	ND	NE	2.0	0.3	0.2	ND	ND	ND	ND	NE
8	2.1	1.5	0.9	ND	ND	ND	ND	NE	2.0	7.2	4.2	ND	ND	ND	ND	NE
12	2.5	1.6	0.9	ND	ND	ND	ND	NE	2.1	9.8	5.7	ND	ND	ND	ND	NE
18	3.4	1.7	0.9	ND	ND	ND	ND	NE	2.5	13.4	7.4	ND	ND	ND	ND	NE
24	4.4	4.6	2.1	ND	12.4 s2	650	15	7	3.9	17.8	8.4	ND	11.1 s2	600	15	1.8
36	4.5	4.8	2.2	ND	16.3 s2	900	20	9	4.3	24.4	11.1	ND	13.2 s2	1,400	30	2.7
48	4.6	5.0	2.3	ND	16.5 s2	1,900	45	20	4.3	37.5	17.1	ND	13.4 s2	1,900	45	2.6
72	4.6	8.0	3.3	8.2 s	16.7 s2	2,100	50	14	4.4	44.5	20.0	ND	13.7 s2	3,200	70	3.5
96	4.9	7.6	3.6	8.2 s	16.7 s2	3,500	75	23	4.4	52.2	23.4	ND	13.9 s2	4,800	110	4.6
120	4.9	14.4	6.3	9.5 s	18.1 s2	4,900	110	17	4.5	52.5	24.0	ND	16.1 s2	7,500	165	7.1
144	5.0	19.3	8.4	10.4 s	18.9 s2	11,600	255	30	4.7	51.0	22.4	ND	15.2 s2	3,500	75	3.3
168	5.0	14.4	6.3	10.3 s	18.6 s2	8,000	175	28	4.8	42.3	18.4	ND	14.7 s2	3,700	80	4.3
192	5.1	10.9	4.7	9.0 s	17.8 s2	6,000	130	28	4.8	40.5	17.6	ND	14.1 s2	2,700	60	3.4

^a Cultures were grown in SCGR at 30°C.

^b Gene expression was induced at time zero.

^c Calculated by using an NCI-ELISA using specific polyclonal antibodies. ND, no bacteriocin detected.

^d Antimicrobial activity against *P. damnosus* CECT4797 as determined by an ADT. Inhibition zones are differentiated as follows: s, sharp; s2, extremely sharp. ND, no inhibition zone detected using 50 µl of supernatant (S) or 20-fold-concentrated supernatant (CS).

^e Antimicrobial activity of CS against *P. damnosus* CECT4797 as determined by an MPA. ND, no inhibition detected using 100 µl of CS.

^f Specific antimicrobial activity, i.e., the antimicrobial activity of CS (BU/mg CDW), calculated by an MPA, divided by the EntL50A or EntL50B concentration (ng/mg CDW). NE, not evaluable.

cation exchange (SP Sepharose Fast Flow) and hydrophobic interaction (Octyl Sepharose CL-4B) chromatographies, followed by reversed-phase chromatography (PepRPC HR 5/5) in a fast-protein liquid chromatography (FPLC) system (GE Healthcare Life Sciences). The antimicrobial activities of the fractions obtained during the purification procedure were determined by an MPA using *P. damnosus* CECT4797 as the indicator microorganism. Fractions displaying a high and specific bacteriocin activity were pulled together and rechromatographed on the same reversed-phase column until chromatographically pure bacteriocin peptides were obtained. The concentrations of EntL50A and EntL50B in the supernatants and the fractions obtained at the end of the purification procedure were determined by an NCI-ELISA.

The purified recombinant bacteriocin peptides were subjected to mass spectrometry analysis by using a matrix-assisted laser desorption ionization–time-of-flight Voyager-DE STR mass spectrometer (MALDI-TOF MS) (PerSeptive Biosystems, Foster City, CA) at the mass spectrum service of the Centro de Genómica y Proteómica, UCM (Madrid, Spain).

Tricine-SDS-PAGE, Western blotting, and gel overlay assay. FPLC-purified EntL50A and EntL50B were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 16% (wt/vol) Tricine gels (Tricine-SDS-PAGE) (42) after silver staining. Western blotting using anti-LR1-KLH (specific for EntL50A) and anti-LR2-KLH (specific for EntL50B) antibodies was performed essentially as described previously (22). To determine the antimicrobial activities of purified recombinant EntL50A and EntL50B, a gel overlay assay (3) was performed using *P. damnosus* CECT4797 as the indicator microorganism.

RESULTS

Cloning of *entL50A* and *entL50B*, separately and together, into the *S. cerevisiae* protein expression and secretion vector pYABD01. The *MFαI_s*-containing pYABD01 vector (6,101 bp) constructed in this work (Fig. 1) was maintained by approximately 100% of the *S. cerevisiae* INVSc1-α(pYABD01) cells grown for at least 50 generations in the absence of selective pressure (results not shown). The bacteriocin structural genes *entL50A* and *entL50B* were cloned, separately and together, into pYABD01, resulting in pYABD02, pYABD03, and

pYABD04, respectively (Fig. 1). Specific PCR amplifications, as well as restriction analysis and DNA sequencing, confirmed the correct construction of the plasmids obtained in this work (results not shown). In the case of the recombinant plasmid pYABD04, several attempts to sequence the fragment α-L50A–α-L50B were made, but the sequencing signal was lost immediately after the last codon of *MFαI_s* fused to *entL50A*. In order to overcome this problem, pYABD04 was digested with HindIII-SmaI and SmaI-XbaI, generating the fragments α-L50A and α-L50B, respectively, which were sequenced and shown to contain the correct sequences.

Heterologous expression and secretion of biologically active EntL50A and EntL50B by *S. cerevisiae*. *S. cerevisiae* L50A(pYABD02) and *S. cerevisiae* L50B(pYABD03) cultures spotted onto YPGR and SCGR plates and grown at 30°C for 30 days showed no direct antimicrobial activity. However, the supernatants and/or CS from cultures of these recombinant strains grown at 30°C in YPGR and SCGR broth for 8 days displayed antimicrobial activity (results not shown). It is pertinent to note that no antimicrobial activity was found in the supernatants and CS from control cultures of *S. cerevisiae* INVSc1-α(pYABD01) (results not shown), which rules out the possibility that the extracellular antimicrobial activity exerted by *S. cerevisiae* L50A(pYABD02) and *S. cerevisiae* L50B(pYABD03) transformants was due to metabolites other than bacteriocins. The *S. cerevisiae* L50A-20(pYABD02) and *S. cerevisiae* L50B-4(pYABD03) transformants were selected for further microbiological and immunochemical quantification of recombinant EntL50A and EntL50B production, respectively (Table 3). The antimicrobial activities of the supernatants and CS from cultures of *S. cerevisiae* L50A-20(pYABD02) grown in

the minimal medium SCGR were first detected 72 and 24 h after induction of gene expression (at time zero), respectively, and the maximum antimicrobial activity was found after incubation for 144 h (3.7 and 255 BU/mg CDW, respectively) (results not shown and Table 3). Although no antimicrobial activity was detected in the supernatants from *S. cerevisiae* L50B-4(pYABD03) grown in the same medium up to 192 h, the CS of this recombinant strain displayed antimicrobial activity, which was first detected at 24 h of incubation and maximal after 120 h (165 BU/mg CDW). The presence of the recombinant bacteriocin peptides in the supernatants from these transformants and their absence in those from *S. cerevisiae* INVSc1- α (pYABD01) were demonstrated by an NCI-ELISA. The production of EntL50A and EntL50B by *S. cerevisiae* L50A-20(pYABD02) and *S. cerevisiae* L50B-4(pYABD03), respectively, started immediately after induction of gene expression and was parallel to cell growth, and the bacteriocin peptides reached their maximum concentration at the beginning of the stationary phase or at the late stage of the exponential growth phase, respectively (Table 3). In this respect, the maximum concentrations of EntL50A (19.3 ng/ml or 8.4 ng/mg CDW) and EntL50B (52.5 ng/ml or 24.0 ng/mg CDW) in the supernatants from *S. cerevisiae* L50A-20(pYABD02) and *S. cerevisiae* L50B-4(pYABD03) were detected at 144 and 120 h of incubation, respectively. The maximum specific activity of EntL50A in the CS from *S. cerevisiae* L50A-20(pYABD02) (30 BU/ng EntL50A) was about fourfold higher than that of EntL50B in the CS from *S. cerevisiae* L50B-4(pYABD03) (7.1 BU/ng EntL50B). On the other hand, these transformants grew much faster and to a higher cell density in the complex medium YPGR (OD₆₀₀ of 8.5) than in SCGR (OD₆₀₀ of 4.8 to 5.1). However, the extracellular antimicrobial activity of *S. cerevisiae* L50A-20(pYABD02) was lower than that in SCGR and was detected only in the CS, and no antimicrobial activity was found either in the supernatants or in the CS from *S. cerevisiae* L50B-4(pYABD03) (results not shown). The antimicrobial activity and production of EntL50A by *S. cerevisiae* L50A-20(pYABD02) started 48 h after gene expression induction, corresponding to the early stationary phase of growth in YPGR. The maximum antimicrobial activity (diameter of inhibition zone of 9.3 mm) and EntL50A concentration (15.0 ng/ml or 5.2 ng/mg CDW) were detected after incubation for 96 h. Surprisingly, notwithstanding the fact that *S. cerevisiae* L50B-4(pYABD03) did not show antimicrobial activity in YPGR, EntL50B was detected in the corresponding supernatants and reached its maximum concentration (56.3 ng/ml, corresponding to 19.1 ng/mg CDW) after 6 days of incubation. Moreover, it is interesting to note that the antimicrobial activity of these EntL50A and EntL50B concentrations was lower than that exerted by similar peptide concentrations (3.6 to 6.3 and 17.1 to 20.0 ng/mg CDW, respectively) produced in SCGR (diameters of 16.7 to 18.1 and 13.4 to 13.7 mm, respectively) (Table 3).

The simultaneous production of EntL50A and EntL50B by a single yeast strain was investigated using *S. cerevisiae* L50AB (pYABD04) transformants. As in the case of *S. cerevisiae* L50A (pYABD02) and *S. cerevisiae* L50B (pYABD03) transformants, no direct antimicrobial activity was detected in cultures spotted onto SCGR plates and grown for up to 30 days (results not shown). However, bacteriocin production was detected and

quantified by antimicrobial and immunochemical assays in the supernatants and CS from cultures of the selected transformant *S. cerevisiae* L50AB-2(pYABD04) grown in SCGR broth for 8 days. The antimicrobial activities of the supernatants and CS were first detected 24 and 8 h after induction of gene expression (at time zero), respectively, and the maximum antimicrobial activities were found after incubation for 144 h (4.4 and 303 BU/mg CDW, respectively). The maximum EntL50A concentration and specific activity in the supernatants and CS of *S. cerevisiae* L50AB-2(pYABD04) were 24 ng/ml (10.4 ng/mg CDW) and 29 BU/ng EntL50A, respectively, but strikingly, EntL50B was not produced.

In order to determine whether EntL50A and EntL50B could be produced in a mixed culture, *S. cerevisiae* L50A-20 (pYABD02) and *S. cerevisiae* L50B-4(pYABD03) were cocultured in SCGR broth at a 1:1 ratio at 30°C for 8 days. Although growth was similar to that obtained when yeasts were grown independently, the antimicrobial activity of the nonconcentrated coculture supernatants was detected earlier and its maximum value was higher (Table 4). In this respect, the maximum antimicrobial activity of the supernatants, found after 96 h of incubation, was 170 BU/mg CDW, corresponding to 10.3 ng/ml (4.7 ng/mg CDW) of EntL50A and 46.4 ng/ml (21.7 ng/mg CDW) of EntL50B. Interestingly, the independent antimicrobial activities of similar amounts of EntL50A (3.6 to 6.3 ng/mg CDW) and EntL50B (20 to 24 ng/mg CDW) in the CS from *S. cerevisiae* L50A-20(pYABD02) and *S. cerevisiae* L50B-4 (pYABD03), respectively, were 70 to 110 BU/mg CDW. These results demonstrate the production of both EntL50A and EntL50B by the coculture and suggest that the recombinant bacteriocin peptides act synergistically. To further confirm and quantify this synergistic effect, supernatants from *S. cerevisiae* L50A-20(pYABD02) and *S. cerevisiae* L50B-4(pYABD03) were challenged against *P. damnosus* CECT4797 independently and mixed in a 1:1 bacteriocin peptide ratio. As expected, the equimolar mixture of EntL50A and EntL50B exerted a greater antimicrobial effect (133 BU/mg CDW) than the additive effect of the peptides acting independently (53 and 23 BU/mg CDW, respectively), demonstrating a degree of synergy of approximately 1.8-fold (Fig. 2).

Purification and characterization of EntL50A and EntL50B heterologously produced by *S. cerevisiae* L50A-20(pYABD02) and *S. cerevisiae* L50B-4(pYABD03), respectively. The specific antimicrobial activities of purified EntL50A and EntL50B were 452- and 2,000-fold greater than those of culture supernatants from *S. cerevisiae* L50A-20(pYABD02) and *S. cerevisiae* L50B-4(pYABD03) grown in SCGR broth at 30°C for 144 and 120 h, respectively. Interestingly, although the antimicrobial activity in these fractions represented a recovery of only 1.3 and 2.6% of the EntL50A and EntL50B original activity, respectively, the corresponding amounts of recombinant EntL50A and EntL50B were 8.6 and 23.5 μ g, respectively, which represent 46.5 and 45.8% of the original bacteriocin peptide amounts, respectively (Table 5). The purity and molecular mass of recombinant EntL50A and EntL50B were analyzed by MALDI-TOF MS. The results obtained for recombinant EntL50A revealed three major peptides with molecular masses ranging from 6.2 to 6.4 kDa (Fig. 3A), while those for recombinant EntL50B showed two major peptides with molecular masses (ca., 5,242 and 5,226 Da) closely similar to that of

TABLE 4. Production and antimicrobial activity of recombinant EntL50A and EntL50B from an *S. cerevisiae* L50A-20(pYABD02) and *S. cerevisiae* L50B-4(pYABD03) coculture^a

Time (h)	OD ₆₀₀ ^b	Bacteriocin peptide concn in ^c :				Antimicrobial activity as assessed by:			
		ng/ml		ng/mg CDW		ADT (mm) ^d	MPA in ^e :		
		EntL50A	EntL50B	EntL50A	EntL50B		BU/ml	BU/mg CDW	
0	1.9	ND	ND	ND	ND	ND	ND	ND	
24	4.0	3.3	20.5	1.6	9.7	7.9 s	40	20	
48	4.2	6.8	42.0	3.1	19.1	8.1 s	70	30	
72	4.3	8.8	44.3	4.0	20.0	10.2 s	310	140	
96	4.4	10.3	46.4	4.7	21.7	10.7 s	360	170	
120	4.4	9.4	55.3	4.2	24.8	10.3 s	320	140	
144	4.5	8.4	45.7	3.7	20.4	9.8 s	260	120	
168	4.5	7.1	36.0	3.2	16.1	9.3 s	210	90	

^a *S. cerevisiae* L50A-20(pYABD02) and L50B-4(pYABD03) were grown at a 1:1 ratio in SCGR broth at 30°C.

^b Gene expression was induced at time zero.

^c EntL50A and EntL50B concentration calculated by an NCI-ELISA using specific polyclonal antibodies for EntL50A or EntL50B, and expressed as ng/ml and ng/mg CDW. ND, no bacteriocin detected.

^d Antimicrobial activity against *P. dammosus* CECT4797 as determined by an ADT. Inhibition zones are differentiated as in Table 3. ND, no inhibition zone detected using 50 μ l of supernatant.

^e Antimicrobial activity of supernatant against *P. dammosus* CECT4797 as determined by an MPA. ND, no inhibition detected using 100 μ l of supernatant.

natural EntL50B (5,178 Da), as well as a minor peptide with the expected molecular mass (5,179 Da) (Fig. 3B). Recombinant EntL50A and EntL50B were also analyzed on silver-stained Tricine-SDS-PAGE gels (Fig. 4A), revealing a major band of the expected size, as well as two and one upper band, in the samples containing EntL50A and EntL50B, respectively, suggesting that both recombinant peptides display a strong tendency to form aggregates. The peptides and peptide aggregates were also detected by Western blotting using antibodies specific for EntL50A and EntL50B (Fig. 4B), and they were shown to be biologically active by a gel overlay assay (Fig. 4C).

DISCUSSION

This study reports for the first time the heterologous expression and secretion of a non-pediocin-like bacteriocin, EntL50 (EntL50A and EntL50B), by a yeast. This was achieved using pYABD01, a segregationally stable expression and secretion vector derived from the *S. cerevisiae* 2 μ m episomal high-copy-number plasmid pYES2. The recombinant plasmid pYABD01 generated in this work contains the P_{GAL1} promoter for high-level inducible protein expression by galactose and the yeast

13-residue signal peptide MF α 1_s, including the Kex2 cleavage site required for processing of fusion proteins during Sec-dependent secretion. The generated recombinant *S. cerevisiae* strains exerted bacteriocinogenic activity in liquid media (SCGR and/or YPGR broth). Interestingly, although yeast growth was higher in YPGR than in SCGR broth, the extracellular antimicrobial activity was much lower in the former medium, with recombinant EntL50A and EntL50B production representing 62 and 80%, respectively, of that found in SCGR broth. Given the high segregational stability of pYABD01 grown in YPD broth without selective pressure, these differences in bacteriocin extracellular concentrations are unlikely to be due to plasmid instability in the cells grown under non-selective conditions (YPGR broth). Strikingly, EntL50A and EntL50B produced in SCGR broth were more active than similar peptide amounts produced in YPGR broth. The lower antimicrobial activity found in the complex medium YPGR broth may be ascribed to one or more of the following factors: (i) the presence of compounds (e.g., salt, proteins, and/or collagen-like materials) interfering with bacteriocin activity (2, 23, 26, 44, 45), (ii) (higher) aggregation of bacteriocin peptide monomers, rendering less active oligomers and/or complexes with medium constituents (14), and/or (iii) higher proteolytic bacteriocin peptide degradation due to a higher cell density and lysis, yielding a higher concentration of vacuolar proteases (2, 14, 23).

The maximum amounts of EntL50A produced by *S. cerevisiae* L50A-20(pYABD02) in SCGR and YPGR broth were approximately three- to fourfold lower, respectively, than those of EntL50B produced by *S. cerevisiae* L50B-4(pYABD03) in the same media. At first glance, these differences may be ascribed to a higher C-terminal proteolytic degradation of EntL50A and/or to a higher secretion of EntL50B. With respect to the latter possibility, it has been demonstrated that the efficiency of secretion partially depends on the nature of the heterologous protein (6, 45); however, we consider it unlikely that this phenomenon is responsible for the differences in EntL50A and EntL50B concentrations found in the respective supernatants, since these peptides show similar physicochem-

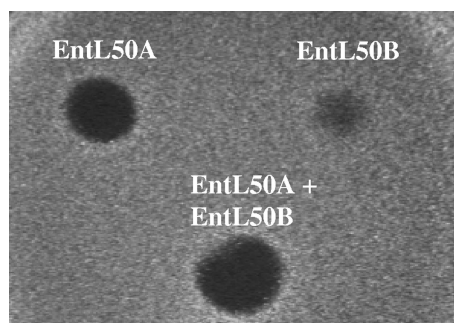


FIG. 2. Antimicrobial activity of supernatants from *S. cerevisiae* L50A-20(pYABD02) (EntL50A) and *S. cerevisiae* L50B-4(pYABD03) (EntL50B) cultures grown in SCGR broth at 30°C, independently and combined to achieve a 1:1 bacteriocin peptide ratio (EntL50A+EntL50B), against *P. dammosus* CECT4797 as determined by a SPAT.

TABLE 5. Purification of recombinant EntL50A and EntL50B produced by *S. cerevisiae* L50A-20(pYABD02) and *S. cerevisiae* L50B-4(pYABD03), respectively^a

Purification stage	Vol (ml)	Total A_{254} ^b	Total activity (BU) ^c	Sp act (BU/ A_{254}) ^d	Increase in sp act (fold) ^e	Total activity (%)	Enterocin yield (ng) ^f	Enterocin yield (%)	Enterocin sp act (BU/ng) ^g
<i>S. cerevisiae</i> L50A-20(pYABD02)									
Culture supernatant	1000	24,580	142,100	6	1	100	18,500	100	7.7
Ammonium sulfate precipitation	100	221	15,700	71	12	11	ND	ND	ND
Gel filtration chromatography	200	37.4	12,800	342	57	9	ND	ND	ND
Cation exchange chromatography	50	3.9	1,100	282	47	0.8	ND	ND	ND
Hydrophobic-interaction chromatography	10	0.8	14,200	17,750	2,958	10	ND	ND	ND
Reversed-phase chromatography	2.6	0.7	1,900	2,714	452	1.3	8,600	46.5	0.2
<i>S. cerevisiae</i> L50B-4(pYABD03)									
Culture supernatant	1000	14,400	46,000	3	1	100	51,300	100	0.9
Ammonium sulfate precipitation	100	157	10,000	64	21	22	ND	ND	ND
Gel filtration chromatography	200	29.6	6,400	216	72	14	ND	ND	ND
Cation exchange chromatography	50	0.5	0	0	0	0	ND	ND	ND
Hydrophobic-interaction chromatography	10	3.0	2,700	900	300	5.9	ND	ND	ND
Reversed-phase chromatography	1.9	0.2	1,200	6,000	2,000	2.6	23,500	45.8	0.05

^a Cultures were grown in SCGR broth at 30°C.

^b Absorbance at 254 nm multiplied by the volume in milliliters.

^c Antimicrobial activity in bacteriocin units per milliliter (BU/ml) as determined by an MPA multiplied by the total volume.

^d Specific activity expressed as the total activity (BU) divided by the total A_{254} .

^e The specific activity of a fraction (BU/ A_{254}) divided by the specific activity of the culture supernatant (BU/ A_{254}).

^f EntL50A and EntL50B concentration as determined by an NCI-ELISA using specific polyclonal antibodies for EntL50A or EntL50B. ND, not determined.

^g Specific activity expressed as the total activity (BU) divided by the enterocin yield (ng).

ical characteristics and are highly related (72% identity, most pronounced at the N terminus), sharing 31 out of 44 and 43 amino acid residues, including the first 8 amino acid residues (8, 10). Moreover, hydrophobic proteins or peptides, such as most bacteriocins, are known to interact with the producer cell membranes (43, 44). In this respect, EntL50A is more hydrophobic than EntL50B (GRAVY [grand average of hydropathy] indexes of 0.202 and -0.144 , respectively) (10), which may lead to a higher retention within the yeast cytoplasmic membrane after an initial pH-dependent cell surface adsorption and thus

to lower EntL50A concentrations in the respective supernatants. In a previous study, we showed that EntL50A and EntL50B are produced in equimolar amounts by the wild-type strain *E. faecium* L50 (14), and therefore, we have evaluated in this work the recombinant paired production of EntL50A and EntL50B by a single *S. cerevisiae* strain. The extracellular production of EntL50A by *S. cerevisiae* L50AB-2(pYABD04) was 24% higher than that by *S. cerevisiae* L50A-20(pYABD02), but strikingly, EntL50B was not produced. Considering the signal loss found during sequencing of the fragment α -L50A- α -L50B

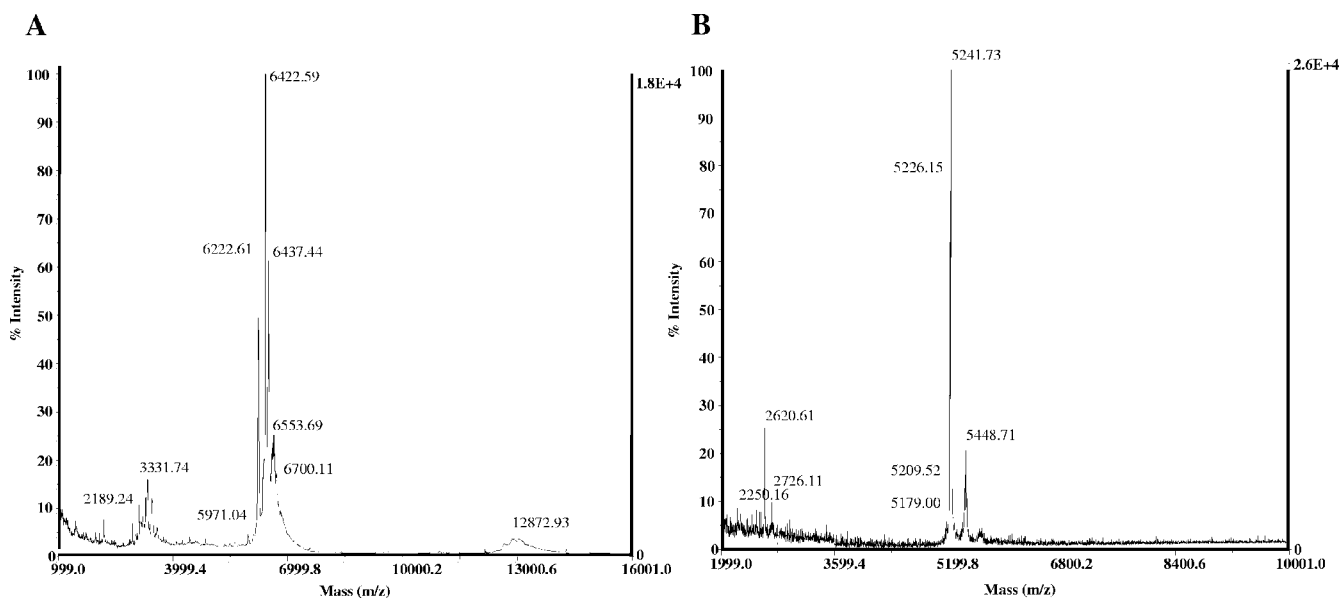


FIG. 3. Mass spectrometry analysis of recombinant EntL50A (A) and EntL50B (B) purified from *S. cerevisiae* L50A-20(pYABD02) and *S. cerevisiae* L50B-4(pYABD03) cultures, respectively, grown in SCGR broth at 30°C.

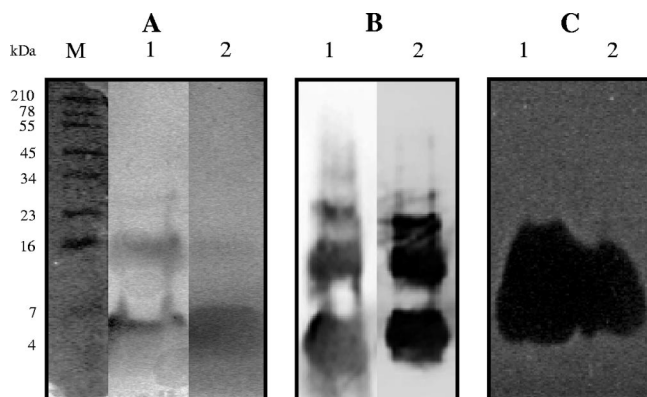


FIG. 4. (A) Tricine-SDS-PAGE of purified recombinant EntL50A and EntL50B after silver staining. (B) Western blotting using rabbit polyclonal antibodies with specificity for EntL50A (anti-LR1-KLH) and EntL50B (anti-LR2-KLH). (C) Antimicrobial activity after gel overlay with the indicator strain *P. damnosus* CECT4797. Lane 1, purified EntL50A; lane 2, purified EntL50B. SeeBlue Pre-Stained Standard molecular mass markers band sizes (M) (Invitrogen) are indicated on the left.

and the high similarity between *entL50A* and *entL50B*, it is likely that DNA secondary structures are established in this fragment and probably in the corresponding mRNA, leading to the repression of *entL50B* expression at the transcriptional and/or translational level and to the higher expression of *entL50A*. In this respect, it is known that stem-loop structures may block polymerase progress (32) and inhibit translational initiation in *S. cerevisiae* (38, 48). Although the paired production of EntL50A and EntL50B by a single *S. cerevisiae* strain was not achieved, we succeeded in the coproduction of both peptides by coculturing *S. cerevisiae* L50A-20(pYABD02) and *S. cerevisiae* L50B-4(pYABD03) and showed that recombinant EntL50A and EntL50B act synergistically at a 1:1 ratio, as reported in previous studies for in vitro-synthesized EntL50A and EntL50B (1, 8). To date, bacteriocin secretion by *S. cerevisiae* has been described only for PedPA-1 (44) and pediocin-like plantaricin 423 (46). Contrary to our results, recombinant strains of *S. cerevisiae* heterologously expressing and secreting PedPA-1 or plantaricin 423, under the control of the alcohol dehydrogenase I gene promoter (P_{ADH1}), showed antimicrobial activity on solid medium. However, only low extracellular bacteriocin activity was obtained in the CS from the recombinant yeasts, likely due to bacteriocin retention within the cytoplasmic membrane and/or adsorption to the producer cells (44, 46).

Notwithstanding the fact that the maximum amounts of EntL50A and EntL50B heterologously produced by *S. cerevisiae* L50A-20(pYABD02) and *S. cerevisiae* L50B-4(pYABD03), respectively, represent only approximately 4 and 11% of the maximum production by *E. faecium* L50 (217 and 210 ng/mg CDW, respectively) (14), the relevance of the strategy developed in this work is highlighted by the fact that it has allowed for the first time the independent purification of these highly related peptides, resulting in a high recovery of purified EntL50A and EntL50B, as determined by using specific antibodies. The MALDI-TOF MS analysis of the last reversed-phase FPLC fraction containing recombinant EntL50B showed a minor peptide with the same molecular mass as natural

EntL50B (5,179 Da), demonstrating that the EntL50B precursor is correctly processed by the Kex2 enzyme, as well as two major peptides with molecular masses of 5,242 and 5,226 Da. These discrepancies may be ascribed to the spontaneous modification of the two methionine residues of EntL50B (i.e., Met¹ and Met²⁴). Regarding this, it is known that Met is the amino acid residue most sensitive to reactive oxygen, resulting in a wide range of peptide and proteins with a reduced biological activity (4, 49). By assuming that both Met residues in recombinant EntL50B have become oxidized to methionine sulfone (MetSO₂), an addition of 64 Da to the theoretical mass, leading to a molecular mass of 5,242 Da, would be obtained. Likewise, the peptide with a molecular mass of 5,226 Da would be the result of the conversion of Met residues to MetSO₂ (addition of 32 Da) and methionine sulfoxide (MetSO) (addition of 16 Da). On the other hand, MALDI-TOF MS analysis of the fraction containing recombinant EntL50A revealed three major peptides with molecular masses of 6.2 to 6.4 kDa. At first glance, this result could indicate that the EntL50A precursor is not correctly processed by the Kex2 enzyme as a consequence of a reduced recognition of the cleavage site (Glu-Lys-Arg) due to a conformational interference exerted by its N terminus; however, the high similarity between EntL50A and EntL50B (most pronounced at the N terminus) does not favor this possibility. Alternatively, recombinant EntL50A may be associated to a hitherto-unknown compound, as has been reported for PedPA-1 heterologously produced by *P. pastoris*, which is tightly associated with some "collagen-like" material, rendering a biologically inactive bacteriocin (2). Although the last reversed-phase FPLC fractions contained approximately 45% of the EntL50A and EntL50B found in the respective culture supernatants, their antimicrobial activities represented only approximately 1 and 3%, respectively, which also favors the bacteriocin oxidation events cited above. In this respect, it has been reported that Met in bacteriocins become spontaneously oxidized, especially during their purification to homogeneity, leading to a loss or reduction of their antimicrobial activities (5, 10, 29). Moreover, a study carried out simultaneously with the one described herein reported the spontaneous chemical modification of EntL50A and EntL50B during their purification due to Met¹ formylation and Met²⁴ oxidation to MetSO, rendering active and inactive peptides, respectively (27). Considering that beer production includes a wort aeration step to promote brewing yeast growth and enhance the start-up of fermentation (47), the oxidative partial inactivation of EntL50A and EntL50B may hamper their application as beer biopreservatives at this stage of the brewing process. Therefore, it would be convenient to develop engineered variants of EntL50A and EntL50B with an increased stability against oxidation by replacing one or both Met residues with other amino acid residues, provided that this replacement is not deleterious for bacteriocin activity, similar to work described for PedPA-1 (29). In this context, replacement of Met at the C-terminal half of bacteriocins (e.g., Met²⁴ in EntL50A and EntL50B) should be carefully addressed, since this region penetrates the hydrophobic part of the target cell membrane, mediating membrane leaking and subsequent cellular death (18, 30, 31).

The results obtained in this work demonstrate the suitability of the generated *MF α* ₁-containing recombinant vector pYABD01 to direct the heterologous secretion of biologically

active EntL50A and EntL50B through the *S. cerevisiae* Sec system. Moreover, these results highlight the feasibility of beer biopreservation by the use of genetically engineered bacteriocinogenic *S. cerevisiae* brewing yeasts as part of a hurdle preservation technology to obtain safer and more stable beers (44, 46, 47). However, despite our promising results, further research is needed to optimize bacteriocin production, increase bacteriocin stability under oxidative stress, and achieve paired production of EntL50A and EntL50B by a single yeast strain. Finally, the development of bacteriocinogenic laboratory and brewing yeast strains overexpressing EntL50 (EntL50A and EntL50B) may facilitate not only the future applications of this broad-spectrum two-peptide bacteriocin as a biopreservative in breweries and other food industries but also the obtention of larger amounts of recombinant bacteriocin peptides to conduct studies such as those on structure-function relationships and molecular mode of action, which are of great applied and scientific interest and remain to be unraveled.

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