

Cloning, Production, and Functional Expression of the Bacteriocin Enterocin A, Produced by *Enterococcus faecium* T136, by the Yeasts *Pichia pastoris*, *Kluyveromyces lactis*, *Hansenula polymorpha*, and *Arxula adeninivorans*

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The bacteriocin enterocin A (EntA) produced by *Enterococcus faecium* T136 has been successfully cloned and produced by the yeasts *Pichia pastoris* X-33EA, *Kluyveromyces lactis* GG799EA, *Hansenula polymorpha* KL8-1EA, and *Arxula adeninivorans* G1212EA. Moreover, *P. pastoris* X-33EA and *K. lactis* GG799EA produced EntA in larger amounts and with higher antimicrobial and specific antimicrobial activities than the EntA produced by *E. faecium* T136.

The enterococci are lactic acid bacteria (LAB) that produce ribosomally synthesized antimicrobial peptides or proteins known as enterocins that attract considerable interest for their potential use as natural and nontoxic food preservatives, for human and veterinary applications, and in the animal production field (17, 26, 34). However, since enterocins may be produced by enterococcal species carrying antibiotic resistance genes and/or genes that code for potential virulence factors, for hygienic, safety, and biotechnological reasons, the production of enterocins in other microbial hosts is being evaluated (9, 20, 21, 22). The enterocin A (EntA) produced by *Enterococcus faecium* T136 (11) is a class IIa bacteriocin with high antilisterial activity (14, 16). The presence of *Listeria monocytogenes* in food can be reduced by *in situ* EntA production (23, 29), by the addition of semi-purified or purified preparations of EntA (1, 24), or by the incorporation of EntA into biodegradable packaging films (31). The heterologous production of EntA by bacterial hosts has been attained through the expression of its native biosynthetic genes (29), by exchange or replacement of the EntA leader peptide and/or dedicated processing and secretion systems (33), or by fusion of mature EntA to signal peptides that act as secretion signals (10, 28, 32). Recently, several yeast platforms have been developed for the large-scale expression of proteins (5, 7, 13, 19). However, the heterologous production of bacteriocins by yeasts has not yet been fully exploited.

Microbial strains, plasmids, and cloning of mature EntA. The microbial strains and plasmids used in this study are listed in Table 1. The primers and inserts used for the construction of the recombinant plasmids are listed in Table 2. For production of EntA by *Pichia pastoris* X-33 and *Kluyveromyces lactis* GG799, derivatives of plasmids pPICZ α A and pKLAC2 were obtained. Primers PKEA-F and PKEA-R were used for PCR amplification from the total genomic DNA of *E. faecium* T136 of a 178-bp XhoI-NotI fragment (KR-EA) carrying the α -factor Kex2 signal protease cleavage site without the Glu-Ala spacer, fused to mature EntA (*entA* without its leader sequence). The resulting XhoI-NotI cleavage fragment was ligated into the pPICZ α A and pKLAC2 vectors to generate plasmids pPICEA and pKLEA, respectively. The presence of the linearized pPICEA and pKLEA plasmids in the transformed *P. pastoris* X-33EA and *K. lactis* GG799EA isolates was confirmed by a

bacteriocinogenicity test, PCR, and sequencing of the inserts. The recombinant plasmids pBTEA and pBYEA were also constructed for the cloning of mature *entA* into *Hansenula polymorpha* KL8-1 and *Arxula adeninivorans* G1212. The primers AHEA-F and AHEA-R were used for PCR amplification from plasmid pPICEA of a 421-bp EcoRI-NotI fragment (MF-KR-EA) carrying the *Saccharomyces cerevisiae* mating pheromone α -factor 1 secretion signal (MF α 1_s) with the Kex2 signal protease cleavage site without the Glu-Ala spacer, fused to mature EntA. The resulting EcoRI-NotI cleavage fragment was ligated into plasmids pBS-TEF1-PHO5 and pBS-AYNI1P-PHO5 to generate intermediate plasmids pBS-TEF1-MF-KR-EA-PHO5 and pBS-AYNI1P-MF-KR-EA-PHO5, respectively. Purified pBS-TEF1-MF-KR-EA-PHO5 was digested with SpeI-SacII, and the resulting 894-bp SpeI-SacII cleavage fragment (TEF1-MF-KR-EA-PHO5) was ligated into plasmid pB25S-ALEU2m to generate plasmid pBTEA. Plasmid pBTEA was further restricted with AscI to remove the *Escherichia coli* part of the plasmid construct, and the yeast ribosomal DNA integrative cassette (YRC) with the selection marker and expression module, flanked by rRNA gene sequences, was used to transform *H. polymorpha* KL8-1 competent cells (7). Purified pBS-AYNI1P-MF-KR-EA-PHO5 was digested with SpeI-SacII, and the resulting 1,187-bp SpeI-SacII cleavage fragment (AYNI1P-MF-KR-EA-PHO5) was ligated into plasmid pB25S-ATRP1m to generate the final plasmid pBYEA. Plasmid pBYEA was further restricted with NcoI, and the resulting YRC was used to transform *A. adeninivorans* G1212 competent cells (7, 8). The presence of the YRCs integrated into *H. polymorpha* KL8-1EA and *A. adeninivorans* G1212EA, respectively, was confirmed by a bacteriocinogenicity test, PCR, and sequencing of the inserts.

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TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Description ^a	Source and/or reference ^b
Strains		
<i>Enterococcus faecium</i>		
T136	EntA and EntB producer; control strain	DNBTA; 11
P13	EntP producer; MPA and ADT indicator	DNBTA; 12
<i>Escherichia coli</i> JM109	Selection of recombinant plasmids	Promega
<i>Pichia pastoris</i> X-33	Yeast wild type	Invitrogen
<i>Kluyveromyces lactis</i> GG799	Yeast wild type	New England Biolabs
<i>Hansenula polymorpha</i> KL8-1	Yeast auxotrophic mutant (<i>aleu2 ura3</i> ⁻)	IPK; 25
<i>Arxula adeninivorans</i> G1212	Yeast auxotrophic mutant (<i>atrp1</i> ⁻)	IPK; 39
Plasmids		
pPICZαA	<i>Zeo</i> ^r ; integrative plasmid carrying the secretion signal sequence from the <i>S. cerevisiae</i> α-factor prepropeptide and functional sites for integration at the 5' <i>AOX1</i> locus of <i>P. pastoris</i> X-33	Invitrogen Life Technologies
pPICEA	pPICZαA derivative carrying the PCR product KR-EA	This work
pKLAC2	<i>Amp</i> ^r ; integrative plasmid carrying the <i>Aspergillus nidulans</i> acetamidase gene (<i>amdS</i>), the secretion signal sequence from the <i>S. cerevisiae</i> α-factor prepropeptide, and functional sites for integration at the <i>lac-4</i> locus of <i>K. lactis</i> GG799	New England Biolabs
pKLEA	pKLAC2 derivative carrying the PCR product KR-EA	This work
pBS-TEF1-PHO5	<i>Amp</i> ^r ; integrative plasmid carrying the constitutive <i>A. adeninivorans</i> -derived P _{TEF1} promoter and the <i>S. cerevisiae</i> -derived <i>PHO5</i> terminator	IPK
pBS-TEF1-MF-KR-EA-PHO5	pBS-TEF1-PHO5 derivative carrying the PCR product MF-KR-EA	This work
pB25S-ALEU2 m	<i>Kan</i> ^r ; integrative plasmid carrying the <i>A. adeninivorans</i> -derived 25S rRNA gene fragment and the <i>ALEU2m</i> gene for auxotrophic complementation	IPK
pBTBA	pB25S-ALEU2m derivative carrying the fragment TEF1-MF-KR-EA-PHO5	This work
pBS-AYNI1-PHO5	<i>Amp</i> ^r ; integrative plasmid carrying the inducible <i>A. adeninivorans</i> -derived P _{AYNI1} promoter and the <i>S. cerevisiae</i> -derived <i>PHO5</i> terminator	IPK
pBS-AYNI1-MF-KR-EA-PHO5	pBS-AYNI1-PHO5 derivative carrying the PCR product MF-KR-EA	This work
pB25S-ATRP1 m	<i>Kan</i> ^r ; integrative plasmid carrying the <i>A. adeninivorans</i> -derived 25S rRNA gene fragment and the <i>ATRP1m</i> gene for auxotrophic complementation	IPK
pBYEA	pB25S-ATRP1m derivative carrying the fragment AYNI1-MF-KR-EA-PHO5	This work

^a ADT, agar well diffusion test; *Zeo*^r, zeocin resistance.

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Antimicrobial activity, ELISA, purification of EntA, and mass spectrometry analyses. The direct antimicrobial activity of the transformed yeasts was screened by a streak-on-agar test (SOAT) (21), and the antimicrobial activity of their supernatants

was examined by a microtiter plate assay (MPA) (22). One bacteriocin unit (BU) was defined as the reciprocal of the highest dilution of the bacteriocin causing 50% growth inhibition (50% of the turbidity of the control culture without bacteriocin) (22). Specific

TABLE 2 Primers, PCR products, and fragments used in this study

Primer, PCR product, or fragment	Nucleotide sequence (5'–3') or description ^a	Purpose
Primers		
PKEA-F	GAATTCCTCGAGAAAAGAACCACCTCATAGTGGAAATATTATGGAAATGG	Amplification of KR-EA
PKEA-R	ATAAGTTGCGGCCGCTATTTAGCACTTCCCTGGAATTGCTCC	Amplification of KR-EA
AHEA-F	AAAGAATTCATGAGATTTCTTCAATTTTACT	Amplification of MF-KR-EA
AHEA-R	TATGCGGCCGCTATTTAGCACTTCCCT	Amplification of MF-KR-EA
PCR products		
KR-EA	178-bp XhoI/NotI fragment containing the α-factor Kex2 signal protease cleavage site fused to mature EntA	Cloning in pPICZαA and pKLAC2
MF-KR-EA	421-bp EcoRI/NotI fragment containing the α-mating factor domain fused to the α-factor Kex2 signal protease cleavage site and mature EntA	Cloning in pBS-TEF1-PHO5 and pBS-AYNI1-PHO5
Fragments		
TEF1-MF-KR-EA-PHO5	894-bp SpeI/SacII fragment containing the constitutive <i>A. adeninivorans</i> -derived P _{TEF1} promoter fused to the α-factor Kex2 signal protease cleavage site, mature EntA, and the <i>S. cerevisiae</i> -derived <i>PHO5</i> terminator	Cloning in pB25S-ALEU2m
AYNI1-MF-KR-EA-PHO5	1,187-bp SpeI/SacII fragment containing the constitutive <i>A. adeninivorans</i> -derived P _{AYNI1} promoter fused to the α-factor Kex2 signal protease cleavage site, mature EntA, and the <i>S. cerevisiae</i> -derived <i>PHO5</i> terminator	Cloning in pB25S-ATRP1m

^a Cleavage sites for restriction enzymes are underlined in the primers. The Kex2 protease processing site is shown in bold.

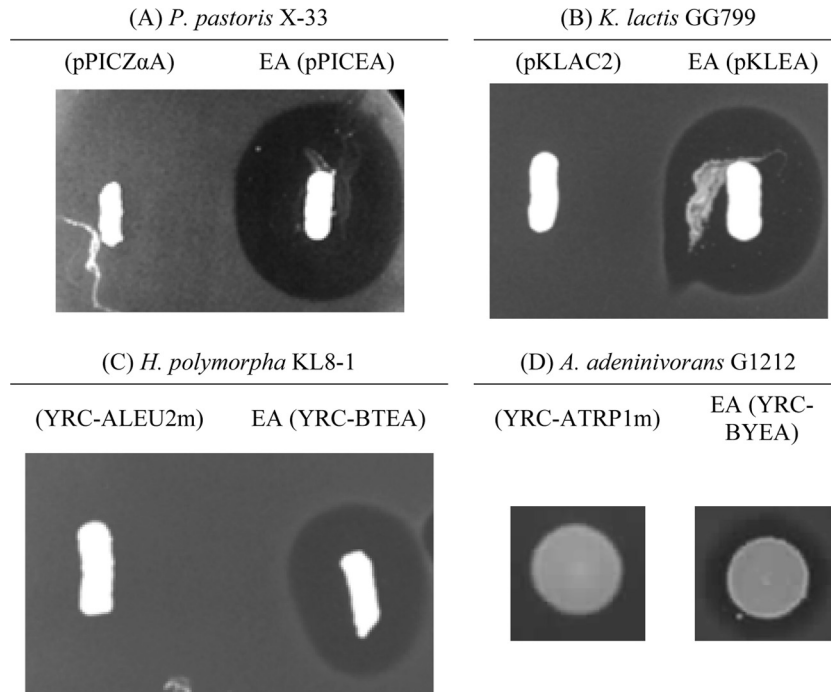


FIG 1 Antimicrobial activities of recombinant yeasts as determined by a SOAT. Panels: A, *P. pastoris* X-33 (pPICZ α A) and X-33EA (pPICEA); B, *K. lactis* GG799 (pKLAC2) and GG799EA (pKLEA); C, *H. polymorpha* KL8-1 (YRC-ALEU2m) and KL8-1EA (YRC-BTEA); D, *A. adenivorans* G1212 (YRC-ATRP1m) and G1212EA (YRC-BYEA). The *P. pastoris* X-33 derivatives were streaked onto the surfaces of plates containing 1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6), 1.34% yeast nitrogen base without amino acids, 4×10^{-5} % biotin, and 0.5% methanol; the *K. lactis* GG799 derivatives were streaked onto plates containing 1% yeast extract, 2% peptone, and 2% galactose; the *H. polymorpha* KL8-1 derivatives were streaked onto yeast extract-peptone-dextrose (Sigma-Aldrich Inc.) plates; and the *A. adenivorans* G1212 derivatives were spotted onto YMM-glucose plates containing 20 mM NaNO₃ (YMM-NO₃) (6). All plates were incubated at 30°C. After growth of the yeast hosts, 40 ml of MRS (Oxoid Ltd.) soft agar containing the indicator microorganism *E. faecium* P13 at about 1×10^5 CFU/ml was poured over the plates, which were then incubated at 30°C overnight for development of halos of inhibition.

polyclonal anti-EntA antibodies and a noncompetitive indirect enzyme-linked immunosorbent assay (NCI-ELISA) were used to detect and quantify the amounts of EntA in the supernatants of the producer yeasts (10). The EntA was purified to homogeneity as previously described (10), and the purified fractions were subjected to matrix-assisted laser desorption ionization–time of flight mass spectrometry (10). The antimicrobial activity of supernatants and their purified EntA were evaluated against *L. monocytogenes* strains from the CECT (Colección Española de Cultivos Tipo, Valencia, Spain) by an MPA.

Heterologous production of EntA by recombinant yeasts.

The *P. pastoris* X-33EA, *K. lactis* GG799EA, *H. polymorpha* KL8-1EA, and *A. adenivorans* G1212EA isolates were selected for their high antimicrobial activity against *E. faecium* P13 (Fig. 1). Yeasts containing control plasmids were used as bacteriocin-negative hosts to disprove that the antagonistic activity of the recombinant producers was due to metabolites other than bacteriocins. The highest production of EntA by *P. pastoris* X-33EA, *K. lactis* GG799EA, and *H. polymorpha* KL8-1EA was 30-, 7-, and 3.2-fold higher, respectively, than the production of EntA by *E. faecium* T136 (Table 3). The P_{AOX1} and P_{LAC4} promoters used in this study are strong inducible promoters to drive the synthesis of heterologous proteins in *P. pastoris* and *K. lactis*, respectively (40, 43). The higher production of EntA by *P. pastoris* X-33EA than by *K. lactis* GG799EA may be due to promoter differences, to the dosages of the *entA* gene in their genomes, and/or to differences in the genetic backgrounds of the two strains. On the other hand, the P_{TEFI}

promoter that drives the production of EntA in *H. polymorpha* KL8-1EA is a strong but constitutive promoter (42). For protein production, inducible systems are often considered superior to constitutive expression systems, since the former enable the achievement of sufficient biomass prior to the initiation of target protein expression and the consequent metabolic burden on the cell (27). The decrease in EntA in the supernatants of *P. pastoris* X-33EA, *K. lactis* GG799EA, and *H. polymorpha* KL8-1EA may be ascribed to, among other factors, the attachment of EntA to cell walls, the formation of aggregates, and/or proteinase degradation, and it may be the subject of further investigations. However, no EntA production by *A. adenivorans* G1212EA was detectable (Table 3). The nitrite reductase promoter (P_{AYNI1}) used for the expression of *entA* in *A. adenivorans* G1212EA is highly repressed by compounds with reduced nitrogen (6), and maybe other factors, such as a lower *entA* gene dosage or deficient MF α 1_s-*entA* recognition by the *A. adenivorans* G1212EA Sec machinery, would explain the low antimicrobial activity and undetectable production of secreted EntA.

The antimicrobial activity of the supernatants of *P. pastoris* X-33EA was 432-fold higher and that of *K. lactis* GG799EA was 59-fold higher than that of *E. faecium* T136, and those of *H. polymorpha* KL8-1EA and *A. adenivorans* G1212EA represented, respectively, 85% and 19% of the antimicrobial activity of *E. faecium* T136. Furthermore, the specific antimicrobial activity of *P. pastoris* X-33EA and *K. lactis* GG799EA was 14.4- and 8.4-fold higher, respectively, than that of *E. faecium* T136 (Table 3). Bacteriocins

TABLE 3 Production and antimicrobial activities of EntA from supernatants of *P. pastoris* X-33EA, *K. lactis* GG799EA, *H. polymorpha* KL8-1EA, and *A. adenivorans* G1212EA

Strain and incubation time (h)	OD ₆₀₀ ^a	EntA production (μg/ml) ^b	Antimicrobial activity (BU/ml) ^c	Specific antimicrobial activity (BU/μg EntA) ^d
<i>P. pastoris</i> X-33EA				
0	0.9	ND	NA	NE
2	1.6	0.5	NA	NE
4	2.8	1.1	31	28
6	3.1	8.4	195	23
8	3.9	14.6	597	41
10	4.4	17.1	1,562	91
12	5.3	22.9	14,910	651
24	6.5	33.0	147,324	4,464
36	6.5	45.1	249,325	5,528
48	6.8	38.6	17,514	454
60	6.9	37.6	14,276	380
72	7.0	21.2	3,156	149
<i>K. lactis</i> GG799EA				
0	1.0	ND	NA	NE
2	1.7	2.0	1,101	551
4	3.4	6.0	6,678	1,113
6	7.6	10.5	34,148	3,252
8	8.4	10.0	29,034	2,903
10	10.2	9.5	22,817	2,402
24	14.6	8.7	9,278	1,066
36	16.6	7.8	7,951	1,019
48	16.0	7.2	6,004	834
60	16.2	6.0	3,083	514
<i>H. polymorpha</i> KL8-1EA				
0	1.0	ND	NA	NE
2	1.4	1.1	75	68
4	1.8	2.0	248	124
6	2.0	2.3	489	213
8	2.7	2.4	207	86
10	2.8	2.5	124	50
12	2.9	3.1	77	35
24	7.2	4.8	26	5
36	7.7	4.1	23	6
48	8.5	3.8	NA	NE
60	8.8	3.6	NA	NE
72	8.4	3.2	NA	NE
<i>A. adenivorans</i> G1212EA				
0	0.1	ND	NA	NE
12	0.8	ND	NA	NE
24	7.0	ND	32	NE
36	24.8	ND	112	NE
48	33.6	ND	86	NE
60	34.6	ND	80	NE
72	46.8	ND	79	NE
84	42.4	ND	82	NE
96	34.6	ND	75	NE
108	38.6	ND	76	NE
<i>E. faecium</i> T136 (16 h)	0.9	1.5	577	385

^a OD₆₀₀, optical density of the culture at 600 nm.^b Production of EntA was calculated by using an NCI-ELISA with polyclonal antibodies specific for EntA. ND, not detected.^c Calculated against *E. faecium* P13 (EntA^s). NA, no activity.^d Calculated as the antimicrobial activity against *E. faecium* P13 divided by the amount of EntA produced. NE, not evaluable. *P. pastoris* X-33EA was grown in broth containing 1% yeast extract, 2% peptone, 100 mM potassium phosphate (pH 6), 1.34% yeast nitrogen base without amino acids, 4×10^{-5} % biotin, and 0.5% methanol; *K. lactis* GG799EA was grown in broth containing 1% yeast extract, 2% peptone, and 2% galactose; *H. polymorpha* KL8-1EA was grown in yeast extract-peptone-dextrose broth; *A. adenivorans* G1212EA was grown in YMM-NO₃ broth (6); and *E. faecium* T136 was grown in MRS broth.^e Most of the data are means from two independent determinations in triplicate.

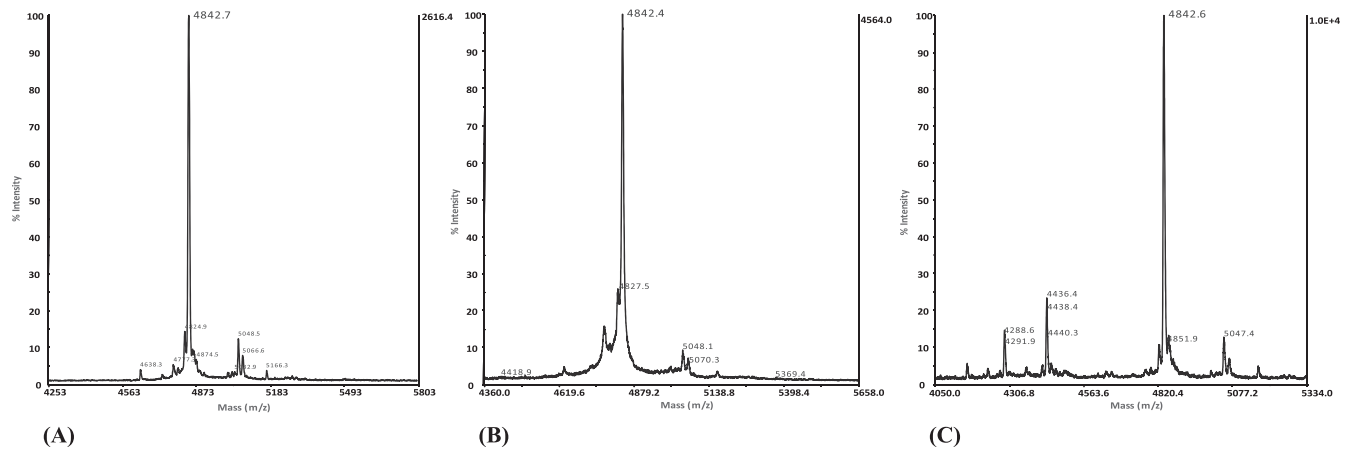


FIG 2 Mass spectrometry analysis of purified EntA from *P. pastoris* X-33EA (A), *K. lactis* GG799EA (B), and *H. polymorpha* KL8-1EA (C).

cloned into recombinant *S. cerevisiae* (2, 37, 41) and *P. pastoris* (3, 4, 21, 36) hosts have been produced with variable success regarding its secretion and functional expression. Recombinant LAB, heterologous producers of EntA, also show higher EntA production (1.5- to 18.5-fold) than *E. faecium* T136, and the EntA they produce shows higher antimicrobial activity (1.5- to 6.6-fold) than that of the EntA produced by *E. faecium* T136, although the specific antimicrobial activity of the EntA produced was lower than that deduced from its production (10, 32). Recombinant LAB, heterologous producers of enterocin P (EntP) and hiracin JM79, also showed higher production and antimicrobial activity of both bacteriocins, whereas their specific antimicrobial activity differed from that of the enterocins produced by the natural enterococcal producers (9, 22, 36). It has been speculated that the lower specific antimicrobial activity of heterologous bacteriocins produced by LAB may be due to, among many other factors, deficiencies in disulfide bond (DSB) formation, a conserved mechanism for stabilizing extracytoplasmic proteins carried out by thiol-disulfide oxidoreductases (9, 10). EntA has two DSBs that seems to improve its antimicrobial activity (15), whereas DSB formation is a prime reason why proteins containing DSBs are difficult to produce in biologically active form by bacterial cell factories (18). An advantage of yeasts over bacterial expression systems is that yeasts may perform posttranslational modifications such as processing of signal sequences, DSB formation, and protein folding in a more efficient way (38, 44). Possibly, the stability of the EntA produced by *P. pastoris* X-33EA and *K. lactis* GG799EA may also be enhanced by the amino acid-rich supplements in the growth medium which may act as alternative and competing substrates for proteinases (30, 35).

Purification of the EntA secreted by *P. pastoris* X-33EA, *K. lactis* GG799EA, and *H. polymorpha* KL8-1EA permitted elevated recovery and high specific antimicrobial activity of the purified bacteriocin. Moreover, the purified EntAs from the recombinant yeasts (Fig. 2) and *E. faecium* T136 (10) showed major peaks with similar molecular masses, suggesting that no different posttranslational modifications occurred. The supernatants of *P. pastoris* X-33EA and *K. lactis* GG799EA and their secreted EntA, purified to homogeneity, showed higher antimicrobial activity against *L. monocytogenes* than that of *E. faecium* T136 (results not shown). Accordingly, *P. pastoris* X-33EA and *K. lactis* GG799EA would be

considered unique yeast cell factories and an alternative to LAB for the heterologous production and recovery of biologically active EntA.

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