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Nisin-Controlled Production of Pediocin PA-1 and Colicin V in Nisin- and Non-Nisin-Producing *Lactococcus lactis* Strains

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The introduction of chimeric genes encoding the fusion leader of lactococcin A-propediocin PA-1 or procolicin V under the control of the inducible *nisA* promoter and the lactococcin A-dedicated secretion genes (*lcnCD*) into *Lactococcus lactis* strains, including a nisin producer, expressing the two component regulator NisRK led to the production or pediocin PA-1 or colicin V, respectively.

The production of the anti-*Listeria* bacteriocin pediocin PA-1 by strains of dairy origin is a desirable objective, since *Listeria monocytogenes* represents a major biological hazard in the dairy industry (19). Although the ability of a recombinant *Lactococcus lactis* starter culture containing a *ped* operon-encoding plasmid to control *L. monocytogenes* in coinoculated cheeses has been demonstrated (1), an alternative approach for the production of pediocin PA-1 in heterologous hosts is based on the amino acid homologies shared by leader peptides of class II bacteriocins (7, 8, 21). Recently, we reported the production of pediocin PA-1 in *L. lactis* as a result of the introduction of a chimeric gene containing a fusion of sequences encoding the lactococcin A leader and mature pediocin PA-1 under the control of the lactococcin A promoter, along with the genes *lcnC* and *lcnD,* which encode the lactococcin A secretion apparatus (12). In this study, the production of pediocin PA-1 in *L. lactis* was enhanced by placing the chimeric structural gene under the control of the nisin-inducible *nisA* promoter (P_{nisA}). A similar strategy was followed to achieve production of the *Escherichia coli* bacteriocin colicin V in *L. lactis*.

Bacterial strains and growth conditions. Lactococcal strains (Table 1) were grown in M17 medium (Oxoid, Unipath Ltd., Basingstoke, United Kingdom) supplemented with 0.5% (wt/vol) glucose (GM17 medium) at 30°C without agitation. *Pediococcus acidilactici* 347 (18) was grown in MRS medium at 30°C without agitation. *E. coli* strains were grown in L broth at 37°C on an orbital shaker. Agar plates were made by adding 1.5% agar to broth media. Antibiotics (Sigma) were added as selective agents when appropriate (chloramphenicol at 5 μg ml^{-1} for lactococci and at 15 μg ml^{-1} for *E. coli,* ampicillin at 200 μg ml^{-1}, and erythromycin at 5 μg ml^{-1}). For induction purposes, nisin A (Aplin and Barret, Trowbridge, United Kingdom) was added to the media used for lactococcal growth at a concentration of 100 ng ml^{-1}.

Construction of pFI2391 and pFI2436. The technique of spliced overlap extension was used in the construction of a P_{nisA}-controlled hybrid gene consisting of a fusion of sequences encoding the lactococcin A leader and mature pediocin PA-1. This technique involved the amplification of two DNA fragments by using the polymerase BIO-X-ACT (Bioline, London, United Kingdom). Primers pAD1 (5′-CCTGAATAATATAG AGATAGGTT-3′) and pAD3 (5′-AAATTTAATTGATTTT TCATTTTGAGTGCCTCC-3′) were used to amplify a 270-bp fragment (fragment 1) containing P_{nisA}. Plasmid pFI1003 (14) was used as template. The 17 nucleotides forming a tail at the 5′ end of primer pAD3 (underlined) are complementary to the amino-terminal sequence of the lactococcin leader. Primers pAD2 (5′-GGAGGCACTCAAAATGAAAAATCAATTAA ATTT-3′) and ppedD (11) (5′-ACCCCGGGATTGATGCCA GCTC-3′) were used to amplify a 242-bp fragment (fragment 2) comprising the hybrid *L-pedA* gene, a fusion of sequences encoding the lactococcin A leader and mature pediocin PA-1 (11). The template was provided by colonies of *L. lactis* FI9043 (11). Primer pAD2 was designed with a 5′ tail corresponding to sequences within the promoter fragment. These 13 nucleotides (underlined) are complementary to the 3′ end of fragment 1. Fragments 1 and 2 were diluted (1:200) in water, and equal quantities of these fragments were mixed. The mixture was used as the template to amplify a 479-bp fragment with primers pAD1 and ppedD. The fragment was cloned into pCR2.1 (Invitrogen), and its identity was confirmed by nucleotide sequence analysis. Then, it was isolated as an EcoRI fragment and cloned into pTG262 to generate pFI2391. Cotransformation of *L. lactis* FI7847 (3) and FI5876 (5) with pFI2391 and pFI2148 (*lcnCD*) (12) by electroporation (4, 9) generated strains FI9917 and FI10038, respectively.

The same strategy was used in the construction of a P_{nisA}-controlled hybrid gene for colicin V production. Primers pAD2 and p135 (5′-CCCGGGTTATAAACAAACATCACTAAG-3′) were used to amplify a 349-bp fragment (fragment 3) comprising the hybrid *L-cvaC* gene, a fusion of sequences encoding the lactococcin A leader and mature colicin V. The template (a sequence encoding the lactococcin leader and *cvaC*, the colicin V structural gene) was provided by pAT.9, a pCR2.1 derivative

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TABLE 1. Lactococcal strains used in this study

Strain no.	Host	Plasmid	Presence of bacteriocin determinant[a]						Reference or source
			lLpedA	*nLpedA*	*nLcvaC*	*lcnCD*	*nis*	*Δnis*	
	FI5876						+		3
FI10038	FI5876						+		
		pFI2391		+					This study
		pFI2148				+			
FI10054	FI5876						+		
		pFII2436			+				This study
		pFI2148				+			
	FI7847							+	5
FI9917	FI7847							+	
		pFI2391		+					This study
		pFI2148				+			
FI10050	FI7847							+	
		pFI2436			+				This study
		pFI2148				+			
FI9262	FI5876								12
		pFI2126	+						
		pFI2148				+			

[a] A plus sign indicates the presence of a bacteriocin determinant. *lLpedA* contains the hybrid *L-pedA* gene preceded by the lactococcin A promoter, *nLpedA* contains the hybrid *L-pedA* gene preceded by the nisin promoter, *nLcvaC* contains the hybrid *L-cvaC* gene preceded by the nisin promoter, *nis* contains the nisin gene cluster, and Δ*nis* contains the nisin gene cluster with a frameshift mutation in codon 16 of the nisin structural gene impeding nisin biosynthesis (the sequences of the downstream nisin cluster genes are unaffected).

carrying a previous 420-bp PCR product with a fusion between sequences encoding the lactococcin A leader and mature colicin V preceded by the lactococcin A promoter. Fragments 1 and 3 were diluted (1:200) in water, and equal quantities of these fragments were mixed. The mixture was used as the template to amplify a 586-bp fragment with primers pAD1 and p135. The fragment was cloned into pCR2.1 and subcloned as an EcoRI fragment into pTG262 to generate pFI2436. Cotransformation of *L. lactis* FI7847 and FI5876 with pFI2436 and pFI2148 (*lcnCD*) generated strains FI10050 and FI10054, respectively.

The pIL277 derivative pFI2148 is compatible with the pTG262-based vectors. PCR analyses, plasmid profiling, and pediocin bioassays with serial subcultures of *L. lactis* FI10038 demonstrated the stability of both plasmids in the lactococcal hosts for at least 100 generations under selective and nonselective conditions (data not shown).

P_{nisA}-controlled expression of hybrid genes. Plate diffusion bioassays involving three independent cultures and *Enterococcus faecalis* TAB28 (pediocin sensitive, nisin resistant) (13) as an indicator showed that *L. lactis* FI9917 and FI10038 produced pediocin PA-1. In the case of FI9917, the addition of subinhibitory levels of nisin A to the culture media was required. Pediocin PA-1 production was quantified by using a series of pediocin PA-1 standards ranging from 0 to 5 μg ml^{-1}. The concentrations of pediocin PA-1 in FI9917 and FI10038 supernatants were 460 ± 18 and 625 ± 21 ng ml^{-1} (mean ± standard deviation), approximately 21 and 30% of the pediocin produced by *P. acidilactici* 347, respectively. The same cultures were used in microtiter plate assays performed as described by Holo et al. (10), which revealed that the activities of FI9917 (251 ± 11 BU ml^{-1}) (one BU [bacteriocin unit] is defined as the reciprocal of the highest dilution causing 50% growth inhibition of the indicator organism) and FI10038 (414 ± 14 BU ml^{-1}) supernatants against *E. faecalis* TAB28 were approximately 20 and 33%, respectively, of that found in *P. acidilactici* 347 supernatants (1,254 ± 39 BU ml^{-1}). Microtiter bioassays with the indicator *L. lactis* FI9180 (pediocin resistant, nisin sensitive) (12) revealed that FI10038 produced nisin and that the activity level achieved was similar to that of FI5876.

The pediocin production of the *L. lactis* FI10038 construct (625 ng ml^{-1}) represents a significant improvement over the pediocin production of *L. lactis* FI9262 (70 ± 8 ng ml^{-1}), since the only difference between FI10038 and FI9262 is that in the latter the hybrid gene is under the control of the lactococcin A promoter (12). Therefore, we have exploited the NICE expression system (2, 15) to enhance pediocin yield in a nisin-producing *L. lactis* strain.

Broth assays with *L. monocytogenes* L15 SV-1/2 as an indicator were performed to elucidate whether coproduction of nisin and pediocin PA-1 by *L. lactis* FI10038 had a synergistic action. Supernatants were obtained from cultures (1×10^8 CFU ml^{-1}) of *L. lactis* FI5876, *L. lactis* FI10038, and *P. acidilactici* 347, adjusted to pH 6, and filter sterilized. The *P. acidilactici* 347 supernatant was diluted to obtain the same concentration of pediocin found in *L. lactis* FI10038 supernatants. Later, 1 ml of the treated supernatants were added to 10-ml nutrient broth (Difco) cultures of *L. monocytogenes* L15 SV-1/2 containing either 1×10^5 or 1×10^8 CFU ml^{-1}. The cultures were incubated for 3 h at 32°C, and, finally, *Listeria* counts were performed in triplicate with MRS agar plates. The broth assays were performed with three independent sets of cultures. Counts for *L. monocytogenes* cultures were significantly lower in the presence of supernatants of *L. lactis* FI10038 than in the presence of supernatants of *L. lactis* FI5876 or *P. acidilactici* 347 (Table 2). The antimicrobial properties of pediocin PA-1 and nisin A and the beneficial effects of their coproduction (6, 17, 20) are features that can be exploited to extend their potential application in the food industry.

Microtiter plate bioassays involving the colicin V-sensitive indicator organism *E. coli* DH5α showed that FI10050 and FI10054 produced colicin V. The activities of FI10050 (657 ±

TABLE 2. Effects of the addition of different culture supernatants on the growth of *L. monocytogenes* L15 SV-1/2

Challenge supernatant	Count (CFU ml^{-1}) for initial *L. monocytogenes* L15 SV-1/2 concn[a]	
	1×10^5 CFU ml^{-1}	1×10^8 CFU ml^{-1}
None	2×10^6	5×10^8
L. lactis FI15876	2×10^5	1×10^8
L. lactis FI10038	3×10^3	2×10^6
P. acidilactici 347[b]	4×10^4	3×10^7

[a] The count was obtained after incubation for 3 h at 32°C.
[b] The *P. acidilactici* 347 culture supernatant was diluted (1:4) with MRS broth.

15 BU ml^{-1}) and FI10054 (1,010 ± 24 BU ml^{-1}) supernatants were approximately 24 and 36%, respectively, of that (2,740 ± 29 BU ml^{-1}) found in the supernatants of *E. coli* ATCC14763, a colicin V-producing strain (7). In the case of FI10050, prior addition of subinhibitory levels of nisin to the culture media was required. Agar diffusion and microtiter plate bioassays with the indicator *L. lactis* FI9180 (colicin resistant, nisin sensitive) revealed that FI10054 produced nisin at a level similar to that of FI5876.

Colicin V is another good target for heterologous production by using the lactococcin A leader and translocatory machinery (7, 8, 21). Production of colicin V has also been obtained in lactic acid bacteria by using the general protein secretory pathway after replacement of the colicin V leader peptide by the signal peptide of the bacteriocin divergicin A (16, 21). However, coproduction of nisin and colicin V by *L. lactis* FI10054 represents the first example of a host producing one bacteriocin (nisin) with activity against gram-positive bacteria and another (colicin V) that is active against gram-negative bacteria. Work is in progress to extend this strategy for the production of other related and nonrelated peptides of industrial interest.

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