

## Nisin-Controlled Extracellular Production of Interleukin-2 in *Lactococcus lactis* Strains, without the Requirement for a Signal Peptide Sequence<sup>∇</sup>

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**Secretion of the cytokine interleukin-2 (IL-2) was investigated in *Lactococcus lactis* using the secretory machinery of the bacteriocin lactococcin A. Surprisingly, the *lcnCD* transport genes were not essential for mouse IL-2 secretion. Furthermore, expression of a mature mouse IL-2 gene resulted in interleukin secretion without the requirement for a leader sequence.**

In most cases secretion of heterologous proteins and peptides in *Lactococcus lactis* has involved fusion to a signal peptide recognized by the general secretory (Sec) pathway (6, 8, 21). Also, the dedicated secretion systems (10) associated with bacteriocins produced by lactic acid bacteria have been exploited (19). In this case leader peptides serve as recognition signals for the corresponding ABC transporters and are cleaved during export at a specific processing site adjacent to two conserved glycine residues located at positions –1 and –2 (11). The flexibility of ABC transporters for secretion and processing of class II bacteriocins has been reported previously (1, 14, 15, 16, 22). In previous studies we exploited the lactococcin A secretion pathway for lactococcal production of pediocin PA-1 (14, 15) and the *Escherichia coli* bacteriocin colicin V (16). Here we describe lactococcal expression and secretion of the eukaryotic cytokine interleukin-2 (IL-2) using the lactococcin A secretion system under control of the nisin promoter *P<sub>nisA</sub>* (17) and the unexpected observation that mouse IL-2 (mIL-2) can be secreted without the requirement for a signal peptide.

**Construction of pFI2398, a chimeric gene (L-mIL-2) consisting of an in-frame fusion of sequences encoding the lactococcin A leader and mature mIL-2.** Primers pAD1 (5'-CCTGA ATAATATAGAGATAGGTT-3') and pIL2a (5'-CTTGAAGT GGGTGCTCCTCCGTTAGCTTC-3') were used to amplify a fragment containing *P<sub>nisA</sub>* and the part of *lcnA* gene encoding the lactococcin A leader. Plasmid pFI2396 (7) was used as the template. Primers pIL2c (5'-GAAGCTAACGGAGGAGCACCCA CTTCAAG-3') and pIL2f (5'-GGATCCTTATTGAGGGCTTG TTGAG-3') were used to amplify a fragment comprising part of the mIL-2 structural gene. Plasmid pTI-IL2 (20) was used as the template.

Plasmids pFI2398 and pFI2148 were introduced into *L. lactis* strains FI5876 (4, 13), FI7847 (5), and UKLc10 (23) (Table 1).

The growth conditions were the growth conditions used previously (4, 5, 13, 23). Plasmid pFI2148 contains the lactococcin A-dedicated translocatory function genes (*lcnCD*). Strains FI7847 and UKLc10 were induced with nisin (at the optimal concentration, 50 ng ml<sup>-1</sup>; added at an optical density at 600 nm of 0.5), whereas FI5876 was autoinduced due to the presence of a complete nisin biosynthesis gene cluster.

Western blotting of culture supernatants from all three constructs revealed two intense bands consistent with unprocessed L-mIL-2 (19.6 kDa) and processed mIL-2 (17.2 kDa) (Fig. 1A) (L-mIL-2 is a chimeric protein containing the lactococcin A leader and mIL-2). Culture supernatants were assayed for the presence of mIL-2 by slot blot analysis (Fig. 1B), and the concentration of the cytokine was measured by a sandwich enzyme-linked immunosorbent assay (ELISA) (Table 1). For each strain, the intensities of the slot blot bands correlated well with the values obtained by the sandwich ELISA. Interestingly, extracellular production of mIL-2 was also detected in the supernatants from the transformants carrying only pFI2398 (L-mIL-2), suggesting that extracellular production was independent of the lactococcin A *lcnCD* genes. However, in this case only unprocessed L-mIL-2 was observed, and the presence of pFI2148 was associated with a higher concentration of mIL-2 in the culture supernatants than in the equivalent strains lacking this plasmid (Table 1 and Fig. 1B).

**Construction of pFI2584, a plasmid containing the mature mIL-2 sequence under control of *P<sub>nisA</sub>*.** Cytokine secretion was evaluated in the absence of a leader peptide. Primers pAD1 and pIL2n13' (5'-AGCTTGAAGTGGGTGCCATTTTGAGT GCCTCC-3') were used to amplify a fragment encoding *P<sub>nisA</sub>* and a part of mature mIL-2. Plasmid pFI2398 was used as the template. Primers pIL2n15' (5'-GGAGGCACTCAAATGG CACCCACTTCAAGC-3') and pIL2f were used to amplify a fragment comprising a part of the sequence encoding mIL-2. Western blot analysis of culture supernatants and intracellular content samples from FI5876, FI7847, and UKLc10 derivatives transformed with pFI2584 revealed that the anti-mIL-2 polyclonal antibodies specifically recognized a 17.2-kDa band, which is the expected size for mIL-2 (Fig. 2A). The ELISA

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TABLE 1. Lactococcal strains used in this study

Strain	Host	Plasmid	Presence of gene(s) <sup>a</sup>							mIL-2 concn (pg/ml) <sup>b</sup>	Reference or source
			L-mIL-2	mIL-2	<i>usp</i> -mIL-2	<i>lcnCD</i>	<i>nis</i>	$\Delta$ <i>nis</i>	<i>nisRK</i>		
	FI5876							+		0	6
	FI7847								+	0	8
	UKLc10								+	0	29
FI9955	FI5876	pFI2398	+					+		160	This study
FI9957	FI5876	pFI2398 pFI2148	+			+		+		215	This study
FI9947	FI7847	pFI2398	+						+	150	This study
FI9949	FI7847	pFI2398 pFI2148	+						+	180	This study
FI10537	UKLc10	pFI2398	+						+	195	This study
FI10538	UKLc10	pFI2398 pFI2148	+			+			+	355	This study
FI10585	FI5876	pFI2584		+				+		175	This study
FI10584	FI7847	pFI2584		+					+	140	This study
FI10586	UKLc10	pFI2584		+					+	285	This study
FI10608	FI5876	pFI2593			+			+		490	This study

<sup>a</sup> L-mIL-2 contains the *lcnA* leader and mIL-2 genes preceded by the nisin promoter; mIL-2 contains the mIL-2 gene preceded by the nisin promoter; *usp*-mIL-2 contains the *usp*<sub>45</sub> leader and mIL-2 genes preceded by the nisin promoter; *nis* is the nisin gene cluster;  $\Delta$ *nis* is 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 not affected); *nisRK* is the *nisRK* genes integrated into the fivefold peptidase-deficient mutant IM16, a derivative of *L. lactis* MG1363.

<sup>b</sup> The concentration of mIL-2 (pg ml<sup>-1</sup>) in culture supernatants of lactococcal strains was determined by a sandwich ELISA. The values are the means from quadruplicate samples (standard deviation,  $\leq$ 8%).

confirmed that there was extracellular production of mIL-2 (Table 1).

Previously, Steidler et al. (20) reported mIL-2 secretion using the *usp*<sub>45</sub> signal peptide, but they observed the presence

of a single 20.1-kDa band. A pTG262 derivative plasmid (pFI2593) carrying the lactococcal *usp*<sub>45</sub> signal peptide fused with the sequence encoding mature mIL-2 under the control of the nisin promoter was engineered and introduced into *L. lactis* FI5876 (strain FI10608). Western blot analysis of con-

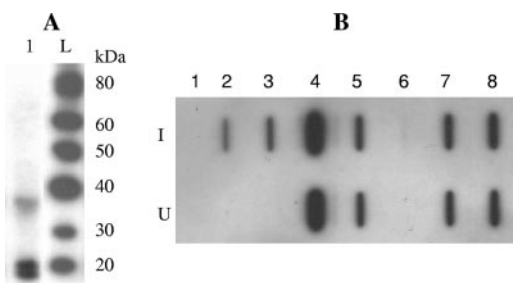


FIG. 1. (A) Detection of mIL-2 in culture supernatant of *L. lactis* FI9957 by Western blot analysis. Lane 1, FI9957; lane L, MagicMark Western protein standard. (B) Slot blot detection of mIL-2 in the culture supernatants of *L. lactis* strains. Row I, nisin induced (50 ng ml<sup>-1</sup>); row U, not induced with exogenous nisin. Lane 1, FI7847; lane 2, FI9947; lane 3, FI9949; lane 4, murine IL-2 (2 ng ml<sup>-1</sup>); lane 5, murine IL-2 (0.2 ng ml<sup>-1</sup>); lane 6, FI5876; lane 7, FI9955; lane 8, FI9957.

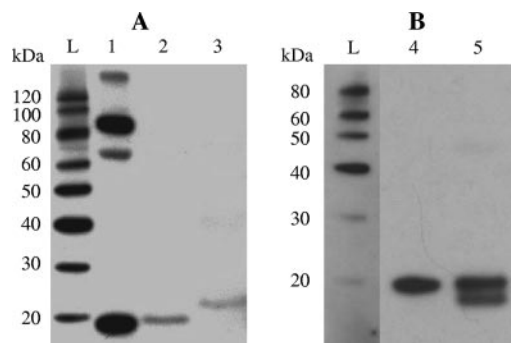


FIG. 2. Western blot detection of mIL-2 in culture supernatants. Lane 1, mIL-2; lane 2, *L. lactis* FI10586; lane 3, *L. lactis* FI10537; lane 4, intracellular contents of *L. lactis* FI10608; lane 5, culture supernatant of *L. lactis* FI10608; lane L, MagicMark Western protein standard.

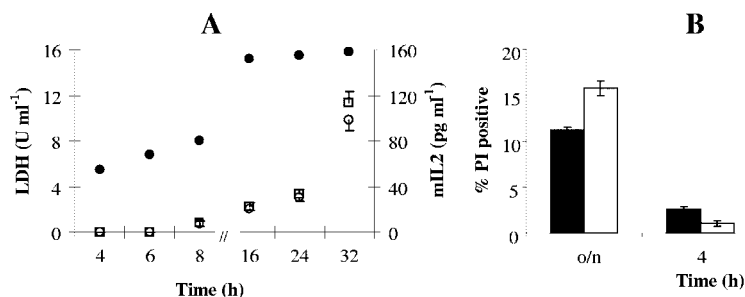


FIG. 3. (A) LDH activity in culture supernatants of *L. lactis* FI10585 (○) and *L. lactis* FI5876(pTG262) (□) and mIL-2 concentrations in the supernatant of *L. lactis* FI10585 (●). (B) Flow cytometric analysis of PI-stained bacteria during different phases of growth. PI permeability is expressed as the percentage of PI-positive events compared to the total number. The error bars indicate standard deviations of three biological replicates. Filled bars, *L. lactis* FI10585; open bars, *L. lactis* FI5876(pTG262).

centrated culture supernatants of *L. lactis* FI10608 revealed two distinct bands corresponding to processed and unprocessed mIL-2 (Fig. 2B), as observed when the *lcnA* leader was used (Fig. 1A).

The CTLL-2 assay described by Gillis et al. (9) and modified by Hopkins and Failla (12) was used to evaluate mIL-2 activity. The values obtained after addition of supernatants from *L. lactis* derivative strains to the CTLL-2 cells agreed with the results obtained with the sandwich ELISA. In addition, we demonstrated that *L. lactis* strains with complete nisin biosynthesis gene clusters (FI9955, FI9957, and FI10585) coproduced nisin A (6.5 to 7.9  $\mu\text{g ml}^{-1}$ ) and mIL-2.

To our knowledge, this is the first report of secretion of a eukaryotic peptide in *L. lactis* assisted by the secretory machinery of a bacteriocin. The supernatants of the transformed strains contained a mixture of both processed and unprocessed mIL-2, which has not occurred previously using this system (7, 16). The lactococcal secretory machinery encoded by the *lcnCD* genes was not necessary for secretion of unprocessed mIL-2 when this peptide was fused to the lactococcal A leader. As a result, transformation of the three lactococcal hosts with a plasmid (pFI2584) containing only the sequence of mature mIL-2 without any signal peptide under the control of  $P_{nisA}$  led to some extracellular production of mIL-2. A number of proteins secreted by bacteria without leader sequences have been reported previously (2), but to date such secretion has involved only noneukaryotic proteins. Proteins using this nonclassical secretion pathway can be predicted using methods such as the method described at the SecretomeP 2.0 website (<http://www.cbs.dtu.dk/services/SecretomeP>). This method gives FASTA protein sequences scores between 0 and 1, and a score greater than 0.5 is considered indicative of nonclassical secretion. Using this system to test the mIL-2 protein sequence resulted in a score of 0.875 when it was tested in the mammalian category, which suggests that mIL-2 might be secreted in eukaryotic cells without the requirement for a signal peptide.

There is evidence that several proteins secreted by nonclassical systems are also present at high intracellular concentrations (3). This observation suggests that when some proteins are abundant in the cytoplasm, they may actually leak out of the cell. In this study, significant mIL-2 levels were detected intracellularly as well as extracellularly. To evaluate cell leakage, the release of cytoplasmic lactate dehydrogenase (LDH) into culture supernatant was investigated using a modified

version of the method of Wittenberger and Angelo (24). The results for LDH activity measured in culture supernatants from *L. lactis* FI10585 did not differ from the results for the parental strain carrying the empty vector (Fig. 3A). The cytokine mIL-2 is smaller than LDH, and the possibility that mIL-2 is released from the cell in a passive way cannot be dismissed.

In order to answer this question, flow cytometry was used to evaluate intact, permeabilized, and lysed (dead) cells in bacterial cultures of FI10585 and FI5876(pTG262). Intact, nisin-induced, and cetyltrimethylammonium bromide-treated bacteria were labeled with thiazole orange and/or propidium iodide (PI) and analyzed by flow cytometry to measure the scatter and fluorescence intensity of individual cells. Thiazole orange labeled all cells, facilitating discrimination of bacteria from the background noise, while cells with permeable membranes were easily distinguished by PI labeling. Samples were taken during different growth phases, and the results showed that in overnight cultures the PI-positive cells accounted for 10 to 15% of the cells of both of the strains (Fig. 3B). After inoculation, the percentage of PI-permeable cells dropped gradually in the exponential growth phase to 1 to 3%, which was also observed at 8 h (data not shown).

Therefore, FI10585 and FI5876(pTG262) showed no significant differences in membrane PI permeability, suggesting the possibility that mIL-2 is secreted without the requirement for a signal peptide by an alternative ABC transporter, as has been reported previously for other secreted leaderless bacterial peptides (18).

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