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Signal Peptide and Propeptide Optimization for Heterologous Protein Secretion in *Lactococcus lactis*

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Lactic acid bacteria are food-grade microorganisms that are potentially good candidates for production of heterologous proteins of therapeutical or technological interest. We developed a model for heterologous protein secretion in *Lactococcus lactis* using the staphylococcal nuclease (Nuc). The effects on protein secretion of alterations in either (i) signal peptide or (ii) propeptide sequences were examined. (i) Replacement of the native Nuc signal peptide (SP_{Nuc}) by that of *L. lactis* protein Usp45 (SP_{Usp}) resulted in greatly improved secretion efficiency (SE). Pulse-chase experiments showed that Nuc secretion kinetics was better when directed by SP_{Usp} than when directed by SP_{Nuc}. This SP_{Usp} effect on Nuc secretion is not due to a better antifolding activity, since SP_{Usp}:Nuc precursor proteins display enzymatic activity in vitro, while SP_{Nuc}:Nuc precursor proteins do not. (ii) Deletion of the native Nuc propeptide dramatically reduces Nuc SE, regardless of which SP is used. We previously reported that a synthetic propeptide, LEISSTCDA, could efficiently replace the native Nuc propeptide to promote heterologous protein secretion in *L. lactis* (Y. Le Loir, A. Gruss, S. D. Ehrlich, and P. Langella, J. Bacteriol. 180:1895–1903, 1998). To determine whether the LEISSTCDA effect is due to its acidic residues, specific substitutions were introduced, resulting in neutral or basic propeptides. Effects of these two new propeptides and of a different acidic synthetic propeptide were tested. Acidic and neutral propeptides were equally effective in enhancing Nuc SE and also increased Nuc yields. In contrast, the basic propeptide strongly reduced both SE and the quantity of secreted Nuc. We have shown that the combination of the native SP_{Usp} and a neutral or acidic synthetic propeptide leads to a significant improvement in SE and in the quantity of synthesized Nuc. These observations will be valuable in the production of heterologous proteins in *L. lactis*.

Gram-positive lactic acid bacteria (LAB) are widely used in food industries for the production and preservation of fermented products. They are considered safe and even beneficial organisms. The potential of using LAB for new applications such as in production of heterologous proteins for biotechnology, in fermented food products, or in the digestive tract of humans or animals is currently under active study (3, 11, 13, 17, 19, 22, 30, 49, 54).

We are focused on optimizing heterologous protein secretion and export in *Lactococcus lactis* (30, 32), a well-characterized LAB for which genetic tools and the genome sequence are available (5, 11). To date, heterologous proteins such as bovine plasmin (3), bovine beta-lactoglobulin (BLG [6a], bovine rotavirus nonstructural protein 4 (NSP4 [13a]), murine interleukin-2 (IL-2) and IL-6 (54), or *Listeria monocytogenes* bacteriophage lysin (17) have been fused to lactococcal signal peptides (SPs) to direct their secretion in the medium. However, secretion efficiency (SE) has been rarely evaluated, and comparison of SE using native or heterologous SP has not been performed. The extent to which these and other features can be refined or improved to optimize protein secretion in *L. lactis* is the subject of this study.

In bacteria, most proteins that are secreted via the Sec pathway are synthesized as precursors containing the mature protein and an N-terminal SP (61) that is essential for precursor secretion. Although the primary sequences are poorly conserved, all SPs display a common tripartite structure including a positively charged N terminus, a hydrophobic core, and a neutral or negatively charged C terminus containing the SP cleavage site (61). Nevertheless, SPs of gram-positive bacteria are longer than those of gram-negative bacteria (61). Therefore, a gram-negative SP may be unable to direct secretion of a protein in a gram-positive host (8). Moreover, in a given species, the SE of a protein can vary with the SP chosen to direct its secretion (40, 47).

Even with the appropriate SP, secretion may be inefficient, and some heterologous proteins remain poorly or not at all secreted, even when fused to a homologous SP (6a, 13a, 46, 47). Notably, the N terminus of the mature moiety may greatly affect the translocation efficiency across the cytoplasmic membrane (2, 32). In *Escherichia coli*, the charge balance between the N termini of the SP and of the mature moiety may be critical for SE (26, 60). Although this charge balance rule was clearly demonstrated for gram-negative bacterial precursors, it may not apply to all gram-positive bacterial and eukaryotic precursors (25, 26). Until now, no detailed investigation was performed on charge balance in protein secretion in LAB.

Some precursors are synthesized as preproproteins, in which the SP is followed by a propeptide that is cleaved after translocation, giving rise to the mature protein (for a review, see reference 50). The propeptides can reportedly influence protein activities as well as SE. The antifolding activity and the role of the long class I propeptides (e.g., propeptides of proteases) in SE have been clearly demonstrated, whereas that of the short class II propeptides, e.g., the *Staphylococcus aureus*

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TABLE 1. Plasmids used in this work

Plasmid	Replicon	Plasmid characteristic(s); cloned *nuc* or *usp45* characteristic(s)	Reference or source
pBS[a]	ColE1	Ap^r	Stratagene
pVE3556	pAMβ1	Em^r, derivative of high-copy-number plasmid pIL253	31
pNZ1011	pUC19	Ap^r, *usp45* gene	58
pBS:Nuc1[b]	ColE1	Ap^r; *nuc* gene expressed from native staphylococcal promoter, P_{staf}	31
pBS:Nuc2[b]	ColE	Ap^r; gene, expressed from P_{staf}, encodes SP_{Nuc}:LEISSTCDA-NucB precursor	31
pBS:Nuc3	ColE1	Ap^r; promoterless *nuc* gene	32
pBS:Nuc4	ColE1	Ap^r; gene, expressed from P_{59}, encodes SP_{Nuc}:LEISSTCDA-NucB precursor	32
pBS:Nuc5	ColE1	Ap^r; DNA encodes SP_{Nuc}:NucT (not expressed)	32
pBS:Nuc6	ColE1	Ap^r; gene, expressed from P_{59}, encodes SP_{Nuc}:NucB precursor	32
pBS:Nuc7	ColE1	Ap^r /Em^r; gene, expressed from P_{staf}, encodes SP_{Nuc}:LGISSTCNA-NucB precursor	This work
pBS:Nuc8	ColE1	Ap^r /Em^r; gene, expressed from P_{staf}, encodes SP_{Nuc}:LQVDDIPSA-NucB precursor	This work
pBS:Nuc9	ColE1	Ap^r /Em^r; gene, expressed from P_{staf}, encodes SP_{Nuc}:LKISSTCHA-NucB precursor	This work
pNuc6	pVE3556	Em^r; gene, expressed from P_{59}, encodes SP_{Nuc}:NucB precursor	32
pNuc9	pVE3556	Em^r; gene, expressed from P_{59}, encodes SP_{Nuc}:NucT precursor	32
pNuc13	ColE1:pVE3556	Ap^r/Em^r; gene, expressed from P_{staf}, encodes SP_{Nuc}:NucB precursor	This work
pNuc14	ColE1:pVE3556	Ap^r/Em^r; gene, expressed from P_{staf}, encodes SP_{Nuc}:LEISSTCDA-NucB precursor	This work
pNuc15	ColE1:pVE3556	Ap^r/Em^r; gene, expressed from P_{staf}, encodes SP_{Nuc}:LGISSTCNA-NucB precursor	This work
pNuc16	ColE1:pVE3556	Ap^r/Em^r; gene, expressed from P_{staf}, encodes SP_{Nuc}:LQVDDIPSA-NucB precursor	This work
pNuc17	ColE1:pVE3556	Ap^r/Em^r; gene, expressed from P_{staf}, encodes SP_{Nuc}:LKISSTCHA-NucB precursor	This work
pBS:U1	ColE1	Ap^r; PCR fragment encoding P_{usp} promoter and SP_{Usp}; amplified with oligonucleotides 5 and 6	This work
pBS:UNuc1	ColE1	Ap^r; PCR fragment encoding SP_{Usp} and *nuc* mature form; amplified with oligonucleotides 7 and 8	This work
pBS:UNuc2	ColE1	Ap^r; gene, expressed from P_{59} encodes SP_{Usp}:NucB precursor	This work
pBS:UNuc3	ColE1	Ap^r; gene, expressed from P_{usp} encodes SP_{Usp}:NucB precursor	This work
pBS:UNuc4	ColE1	Ap^r; gene, expressed from P_{usp} encodes SP_{Usp}:NucT precursor	This work
pBS:UNuc5	ColE1	Ap^r; gene, expressed from P_{usp} encodes SP_{Usp}:LEISSTCDA-NucB precursor	This work
pBS:UNuc6	ColE1	Ap^r; gene, expressed from P_{usp} encodes SP_{Usp}:LEISSTCDA-NucT precursor	This work
pUNuc1	pVE3556	Em^r; gene, expressed from P_{usp}, encodes SP_{Usp}:NucB precursor	This work
pUNuc2	ColE1:pVE3556	Ap^r/Em^r; gene, expressed from P_{59}, encodes SP_{Usp}:NucB precursor	This work
pUNuc3	pVE3556	Em^r; gene, expressed from P_{usp}, encodes SP_{Usp}:LEISSTCDA-NucB precursor	This work
pUNuc4	pVE3556	Em^r; gene, expressed from P_{usp}, encodes SP_{Usp}:NucT precursor	This work
pUNuc5	pVE3556	Em^r; gene, expressed from P_{usp}, encodes SP_{Usp}:LEISSTCDA-NucT precursor	This work
pVE3655	pWV01	Cm^r; carries the nisin-inducible promoter P_{nisA}	P. Langella
pSEC1[c]	pWV01	Cm^r; gene, expressed under P_{nisA}, encodes SP_{Usp}:NucB precursor	6a
pSEC11	pWV01	Cm^r; gene, expressed under P_{nisA}, encodes SP_{Usp}:NucT precursor	This work

[a] pBS, pBluescript II SK(+).
[b] pBS:Nucl and pBS:Nuc2 are referred to as pBS:*nuc* and pBS:*nucmcs*, respectively, and were previously described (31).
[c] pSEC1, previously called pSEC:Muc (6a).

nuclease (Nuc), the *Bacillus amyloliquefaciens* barnase, or the *Bacillus subtilis* amylase, is less studied (39, 57). In *S. aureus*, the Nuc protein containing the propeptide (NucB) is localized in the cell wall, whereas the cleaved mature protein (NucA) is in the medium (9). Nevertheless, this localization is not observed in other hosts such as *L. lactis* or *Corynebacterium glutamicum* (32, 34). Results for *E. coli* demonstrated that the Nuc propeptide slows precursor folding, plays a positive role in SE of a fusion using an *E. coli* SP, and alleviates SecA dependency of precursor secretion in *E. coli* (55).

The secretion capacity of *L. lactis* was previously investigated using Nuc as a secretion reporter (32). Nuc, a small and stable secreted protein, is genetically and biochemically well characterized (51). Its enzymatic activity is readily detectable on petri plates as well as in zymograms (31, 34). Translational fusions to the N terminus and/or the C terminus of the mature protein are enzymatically active (30, 32, 41). SP_{Nuc} is atypical, as it is unusually long (60 residues) and contains two hydrophobic stretches that may form a hairpin in the cytoplasmic membrane during translocation (27). In *L. lactis*, as in *E. coli*, the native Nuc propeptide greatly affects SE. Furthermore, replacement of the native Nuc propeptide by a synthetic one can restore or even enhance SE (32).

Here, we examine the effects of changing the SP and/or propeptide on the secretion and enzymatic activity of the Nuc reporter. We found that the use of the homologous Usp45 SP (SP_{Usp} [58,59]) significantly improves SE. Furthermore, the Nuc propeptide is required for an optimal Nuc SE but can be replaced by synthetic propeptides that are acidic or neutral. The activities and role of charge balance in the enhancement capacity of these propeptides are discussed. The combination of SP_{Usp} and a synthetic propeptide resulted in significant enhancement in SE and also in overall production yields of Nuc. These observations will be valuable in the production of heterologous proteins in *L. lactis*.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. *E. coli* strain TG1 (20) and *L. lactis* strains MG1363 (18) and NZ9000 (28) were used as hosts. Plasmids used are described in Fig. 1 and listed in Table 1. *E. coli* was grown on Luria-Bertani medium (48) and incubated at 37°C. *L. lactis* was grown on M17 medium (56) in which lactose was replaced by 0.5% glucose (M17-glu; Difco) and on brain heart infusion (Difco) and incubated at 30°C. SA medium was used to grow *L. lactis* for pulse-chase experiments (24). Antibiotics were added at the given concentrations: erythromycin, 5 μg/ml for *L. lactis* or 150 μg/ml for *E. coli*; chloramphenicol, 5 μg/ml for *L. lactis* and *E. coli*; and ampicillin, 100 μg/ml for *E. coli*. Induction of the nisin promoter was carried out as follows: an overnight culture was diluted 1:250 into fresh medium and incubated at 30°C until the optical density at 600 nm reached ~0.5. The culture was then divided into two equal volumes, and 1 ng of nisin/ml was added in one tube. The other tube was kept as the noninduced culture control. Cultures were further incubated, and protein samples were prepared after 1 h of induction.

DNA manipulation. Whole-cell lysates were prepared as described previously (44), except that proteinase K was added after lysozyme treatment. This additional proteolytic step eliminates mature Nuc forms associated with protoplasts prior to cell lysis.

Plasmid DNA was isolated essentially as described elsewhere (4), except that, for *L. lactis*, TES buffer (sucrose, 25%; EDTA, 1 mM; Tris-HCl, 50 mM [pH 8]) containing 10 mg of lysozyme/ml was used for 10 min at 37°C to prepare protoplasts. Enzymes were used as recommended by the suppliers. General procedures for DNA manipulations were performed as described elsewhere (32). Electroporation of *L. lactis* was performed as described elsewhere (29), and transformants were plated on M17-glu agar or brain heart infusion agar plates containing the required antibiotic.

Design of synthetic propeptides. PCRs were performed with a Perkin-Elmer Cetus (Norwalk, Conn.) apparatus using *Thermophilus aquaticus* DNA polymerase (Promega) as recommended by the manufacturer. Oligonucleotides were synthesized by MWG Biotech (see Table 2).

To modify the LEISSTCDA synthetic propeptide sequence, a set of oligonucleotides were designed in which acidic residues (glutamate and aspartate) were replaced by neutral (glycine and asparagine, in oligonucleotides 1 and 2, respectively) or basic (lysine and histidine, in oligonucleotides 3 and 4, respectively) residues (see Table 2). The modified oligonucleotides were inserted in *Nsi*I-cut pBS:Nuc1 (31). Both orientations were obtained for each oligonucleotide. When cloned in the noncoding orientation, oligonucleotides 3 and 4 encoded a stop codon and oligonucleotides 1 and 2 encoded a nine-residue propeptide, the sequence of which was LQVDDIPSA. This latter propeptide contained two acidic amino acid residues and was also used to test the effect of negatively charged residues at positions 4 and 5 (instead of 2 and 8). The resulting plasmids are pBS:Nuc7, pBS:Nuc8, and pBS:Nuc9 (listed in Table 1). All constructions were confirmed by DNA sequencing.

To replace SP_{Nuc} by the Usp45 signal peptide (SP_{Usp}), a 291-bp fragment was PCR amplified from pNZ1011 matrix (58) (see Table 1) with oligonucleotides 5 and 6 (see sequences in Table 2). This PCR fragment also contains the *usp45* promoter region (P_{usp}) including 121 bp upstream of the −35 sequence. The reverse primer (oligonucleotide 6) was designed such that an *Nsi*I site was introduced in the last two codons of the fragment. Insertion of an *Nsi*I site allows cloning of fragments encoding the mature Nuc without changing the −2 and −1 residues of SP_{Usp}. This DNA fragment was then cloned on pBluescript (pBS) vector in *E. coli* TG1, resulting in pBS:U1.

An SP_{Usp}:NucB fusion was obtained by joining the *Nsi*I-*Spe*I fragment of pBS:Nuc3 containing the *nuc* mature moiety to *Nsi*I-*Spe*I-cut pBS:U1, resulting in pBS:UNuc3. In pBS:UNuc3, the production of SP_{Usp}:NucB is controlled by the P_{usp} promoter. To test the effect of different promoters on SP_{Usp}:NucB secretion, P_{usp} was deleted from pBS:UNuc3 by PCR amplification using oligonucleotides 7 and 8 (see sequences in Table 2). A 695-bp DNA fragment was generated (*SP_{usp}:nucB*) and cloned into *Sma*I-cut pBS vector in *E. coli* TG1, resulting in pBS:UNuc1.

Derivatives of *SP_{usp}:nuc* fusions. To generate a Nuc derivative devoid of its propeptide, a DNA fragment containing *nucT* (32) was isolated from *Nsi*I-*Xba*I-cut pBS:Nuc5 and cloned into an *Nsi*I-*Xba*I-cut pBS:UNuc3 backbone, resulting in pBS:UNuc5. The NucT mature form contains three positive charges in its first 10 residues (32). A synthetic oligonucleotide encoding LEISSTCDA (32) was inserted into *Nsi*I-cut pBS:UNuc3, resulting in pBS:UNuc5. To generate an SP_{Usp}:LEISSTCDA:NucT fusion, an *Nsi*I-*Xba*I-cut *nucT* fragment was cloned into an *Nsi*I-*Xba*I-cut pBS:UNuc5 backbone, resulting in pBS:UNuc6. These *SP_{usp}:nuc* derivative cassettes were introduced in *L. lactis* MG1363 as *Sac*I-*Xho*I or *Sac*I-*Eco*RI fragments cloned into a *Sac*I-*Xho*I- or *Sac*I-*Eco*RI-digested pVE3556 backbone vector resulting in plasmids pUNuc1 and pUNuc3 (*Sac*I-*Xho*I cloning) or pUNuc4 and pUNuc5 (*Sac*I-*Eco*RI cloning), respectively (Table 1).

High constitutive expression of the SP_{Usp}:NucB precursor was obtained from plasmid pUNuc2, which was constructed as follows. First, the fragment encoding native SP_{Nuc}:NucB on pBS:Nuc6 was replaced by an *SP_{usp}:nucB* cassette isolated from pBS:UNuc1, resulting in pBS:UNuc2. Plasmid pUNuc2 was obtained as a cointegrate of pBS:UNuc2 and pVE3556 joined at the *Sac*I site.

Inducible expression. Nisin-controlled expression is a tightly controlled expression system with high levels of induction (10). Abundant precursor is accumulated using this system, which allowed us to examine the enzymatic activities of the two SP_{Usp}:NucB and SP_{Usp}:NucT precursor forms. The corresponding encoding cassettes were placed under the transcriptional control of the nisin-inducible promoter (P_{nisA}), resulting in plasmids pSEC1 (6a) and pSEC11. For each pSEC plasmid, a *Xho*I-*Bam*HI *SPuspnucB* cassette was cloned into a *Xho*I-*Bam*HI-cut pVE3655 backbone vector, which contains P_{nisA}, followed by a multicloning site. The latter plasmid is a derivative of pNZ8010 (10) (kindly provided by Oscar Kuipers), from which the *gus* gene, expressed from P_{nisA}, was deleted by *Xba*I digestion. Constructions were obtained in *E. coli* TG1 and then established in *L. lactis* NZ9000 (kindly provided by O. Kuipers [28]), a derivative of *L. lactis* MG1363 that carries the *nisRK* regulatory genes.

Expression of novel propeptide fusions to Nuc in *L. lactis*. To test the effect of the different synthetic propeptides on secretion efficiency (the proportion of total protein present in mature secreted form, SE) in *L. lactis*, plasmids pNuc13 to pNuc17 were established in strain MG1363 as cointegrates between *Xba*I-cut pVE3556 and *Xba*I-cut pBS:Nuc1, pBS:Nuc2, pBS:Nuc7, pBS:Nuc8, and pBS:Nuc9. The orientation of the resulting cointegrates was determined by restriction analysis, and plasmids harboring the same backbone structure were selected for further experiments.

Preparation of protein extracts and detection of Nuc fusions by immunoblotting. Protein samples from *L. lactis* cultures were prepared as described previously (32). Briefly, for cell fractionation, 2 ml of *L. lactis* exponential-phase cultures was harvested after a 5-min centrifugation at 6,000 × *g* at 4°C. Cell and supernatant fractions were treated separately. Supernatants were filtered on 0.2-μm-pore-size filters (Millipore, Bedford, Mass.) and precipitated with trichloroacetic acid (15% final concentration). Cell pellets were washed and resuspended in TES, prior to trichloroacetic acid precipitation (10% final concentration). Cell pellets were then washed once with 1 ml of cold acetone, dried, and resuspended in TES containing lysozyme (1 mg/ml; 30 min at 37°C). Cells were lysed with 20% sodium dodecyl sulfate (SDS). Equal volumes of 2× loading buffer were added to all samples. Both supernatant and cell fractions were denatured (5 min at 95°C) prior to SDS-polyacrylamide gel electrophoresis (PAGE).

SE was determined by scanning different nonsaturated film exposures and using the ImageQuant program to get average values. SDS-PAGE, electroblotting on polyvinylidene difluoride membranes (Millipore), and immunoblotting were performed as described elsewhere (32) or according to the manufacturer's recommendations. Rabbit anti-Nuc antibodies were kindly provided by J. R. Miller. Immunodetection was performed with protein G horseradish peroxidase conjugate (Bio-Rad) and an enhanced chemiluminescence kit (Dupont-NEN) as recommended by the suppliers. To evaluate Nuc distribution or to quantitate Nuc SE, several (three to six, depending on construction) independent samples were prepared. Samples to be compared were prepared at the same time and loaded on the same gel. After enhanced chemiluminescence detection, different nonsaturated film exposures were scanned by a Scanjet II (Hewlett-Packard) using Deskscan II and ImageQuant programs and average values were determined. For quantification, signals were compared to those of known amounts of a commercial NucA sample. Both B and A forms of Nuc were included in these estimations.

Pulse-chase conditions. Pulse-chase experiments were performed essentially as described previously (32). An overnight culture of the appropriate *L. lactis* strain grown on SA medium (24) was used to inoculate, at 2%, 20 ml of SA medium with 33.5 μM methionine (Met). Cells were grown at 30°C to an optical density at 600 nm of 0.5; 10 ml of culture was then harvested and washed in SA medium without Met. Cells were resuspended in 2 ml of SA medium without Met and incubated at 30°C for 2 min. Cultures were pulse-labeled for 1 min by the addition of 10 μl of [^{35}S]Met (10 mCi/ml). Seven hundred microliters of Met (5%; 2,500,000-fold excess) was added (chase), and 250-μl samples were taken at given time intervals. Samples were prepared and immunoprecipitated as previously described (32, 37).

Nuc plate activity assays and zymogram. Nuc plate assays were performed as described previously (31). Nuc enzyme activity was evaluated on zymograms. After SDS-PAGE, protein samples were renatured as described elsewhere (34). A 2-mm toluidine blue-DNA agar (TBD-agar) layer was poured in the vertical support used for the polyacrylamide gel (PAG) (Protean II; Bio-Rad). After polymerization, the TBD-agar layer is kept on a single glass plate and dried at 55°C for 30 min. The renatured PAG is then placed on the TBD-agar layer covered with plastic film and incubated at 37°C for 1 to 5 h, depending on the amount of Nuc protein that was loaded in the PAG. After the appearance of bands corresponding to Nuc activity, the zymogram is photographed. Note that the SDS-PAG can be stained with Coomassie blue prior to renaturation treatment. The staining remains after renaturation and does not prevent visualization of Nuc activity (data not shown).

RESULTS

Replacement of SP_{Nuc} by the lactococcal SP_{Usp} enhances Nuc secretion in *L. lactis*. Nuc secretion in *L. lactis* driven by the SP_{Nuc} signal is inefficient (32). We therefore tested the effect of replacing SP_{Nuc} by SP_{Usp}, the SP of the major *L. lactis* secreted protein Usp45 (58). SP_{Usp} comprises 27 residues and

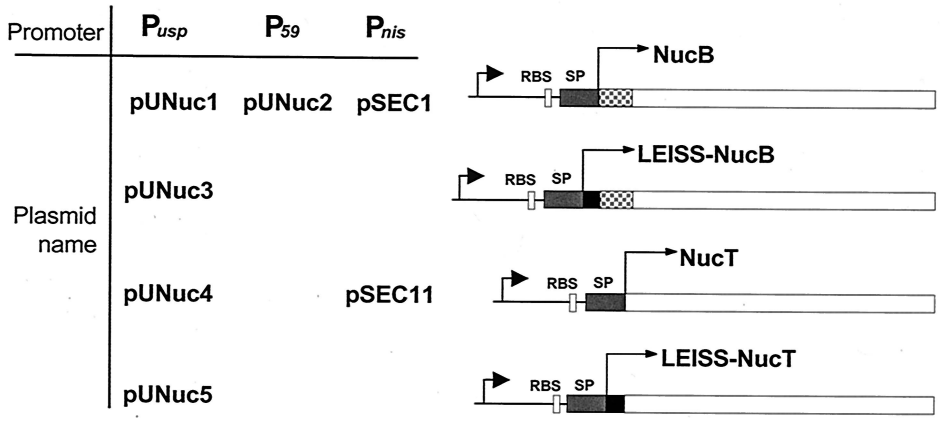

FIG. 1. Expression cassettes for Nuc production and export using the host SP_{Usp}. Schematic structures of fusion proteins (right panel) expressed under the indicated promoters and carried by the indicated plasmids (left panel) are shown. For details of plasmid construction, see the text and Table 1. Black arrowheads indicate *L. lactis* promoter sequences of either the *usp45* gene (P_{usp}), the strong promoter (P_{59}), or the nisin-inducible promoter (P_{nis}). RBS, ribosome binding site of the *usp45* gene; gray bar (SP), Usp45 SP coding region; gray checkered bar, native propeptide coding region; black bar, LEISSTCDA synthetic propeptide coding region; open bar, NucB or NucT coding sequence (not to scale).

is typical of gram-positive bacterial SPs (59). The SP_{Usp}:NucB fusion was constructed and expressed from the P_{usp} promoter (plasmid pUNuc1) (Table 1 and Fig. 1). Western blotting was performed to compare secretion of SP_{Usp}:NucB to that of SP_{Nuc}:NucB (the native Nuc protein is encoded by pNuc6) (data not shown). SE was around 95% with SP_{Usp}, compared to 60% with the native SP_{Nuc}. However, in these experiments, expression was driven by different-strength promoters (P_{usp} on pUNuc1 is weaker than P_{59} on pNuc6); Northern blotting confirmed that Nuc expression from pUNuc1 was about eightfold lower than that observed from pNuc6 (data not shown). To test whether improved SE of SP_{Usp}:NucB was due to lower-level Nuc expression on pUNuc1, expression was ensured by the P_{59} promoter (on plasmid pUNuc2) (Table 1 and Fig. 1). Nuc secretion was also very efficient with this construction compared to that of SP_{Nuc}:NucB (Fig. 2). Note that, in Western experiments, some mature NucA is found associated with the cell fraction (in the cell wall), as already shown and discussed by Dieye et al. (12) and Liebl et al. (34), in *L. lactis* and *C. glutamicum*, respectively. Immunodetection of *L. lactis* Usp45 protein on these samples showed no accumulation of intracellular precursor, indicating that Usp45 secretion was not altered by high-level secretion of Nuc driven by a common SP (data not shown). These results show that replacement of SP_{Nuc} by SP_{Usp} leads to efficient secretion of Nuc even at high expression levels.

SP_{Usp}:NucB is more efficiently processed than SP_{Nuc}:NucB. Processing of the precursors SP_{Usp}:NucB and SP_{Nuc}:NucB was analyzed by pulse-chase labeling experiments using [^{35}S]Met (Fig. 3). The effect of SP replacement on SE in SA, the medium used for pulse-chase labeling, was found to be comparable with that observed in rich medium (data not shown). The proportions of precursor and mature NucB in pulse-labeled SP_{Nuc}:NucB and SP_{Usp}:NucB expressed from pNuc6 and pUNuc2, respectively, were comparable at time zero and 1 min after the chase. SP_{Nuc}:NucB was still present but at decreasing concentrations at 5, 10, and 20 min. In contrast, SP_{Usp}:NucB was detected in only trace amounts at 5 min and was absent at 10 and 20 min. These results are consistent with the conclusion that SP_{Usp}:NucB is more efficiently processed in *L. lactis* than is SP_{Nuc}:NucB.

The antifolding activity of SP_{Usp} is not better than that of SP_{Nuc}. The more efficient processing of SP_{Usp}:NucB could be due to an antifolding activity of SP_{Usp} higher than that of SP_{Nuc} (intramolecular feature). The antifolding activities of the SPs were compared in vitro by means of a Nuc activity assay (zymogram). Constructs in which expression is driven by a nisin-inducible promoter were used to achieve high-level accumulation of the SP_{Usp}:Nuc(B or T) precursor forms (Table 1). Using this system, SP_{Nuc}:Nuc(B or T) and SP_{Usp}:Nuc(B or T) forms were detected in cell extracts by Western blotting (Fig. 4A). Enzymatically active forms were examined by zymograms on samples run on an SDS-PAG. No activity was detected for the precursors SP_{Nuc}:NucB and SP_{Nuc}:NucT (Fig. 4B) even after long exposure of zymograms, thus suggesting that precursor SP_{Nuc}:Nuc(B or T) is inactive in vitro. Similar results were already obtained with SP_{Nuc}:NucB produced from pNuc6 (32). In contrast, Nuc activity bands were detected for both SP_{Usp}:NucB and SP_{Usp}:NucT (Fig. 4B). Although amounts of precursor SP_{Usp}:Nuc(B or T) and SP_{Nuc}:Nuc(B or

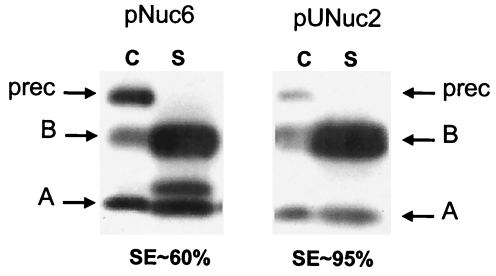

FIG. 2. Replacement of SP_{Nuc} by SP_{Usp} improves Nuc SE. Nuc SE was estimated by Western blot analysis on exponential-phase cultures of lactococcal strains containing pNuc6 (encoding SP_{Nuc}:NucB) and pUNuc2 (encoding SP_{Usp}:NucB). Migration positions of precursor forms (prec) or mature forms of both NucA (A) and NucB (B) are indicated by arrows. C, cell lysates; S, supernatant fraction; SE, the proportion of total protein present in mature secreted form.

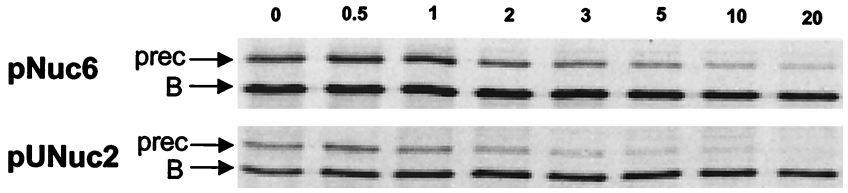

FIG. 3. Comparison of kinetics of SP_{Nuc}:NucB and SP_{Usp}:NucB precursor processing by pulse-chase experiments. MG1363 containing pNuc6 (encoding SP_{Nuc}:NucB precursor) (upper panel) or pUNuc 2 (encoding SP_{Usp}:NucB precursor) (lower panel) was grown in SA medium (24) and pulse-labeled with [^{35}S]Met for 1 min. Samples were taken at different times after the pulse as indicated (in minutes). Time zero corresponds to a sample taken just at the end of the pulse. Migration positions of the different Nuc species are indicated by arrows, prec, precursor; B, NucB.

T) in cell fractions are comparable as revealed by the Western blot, only precursors comprising SP_{Usp} are active in zymograms. Nevertheless, the precursor forms show very weak activity compared to that of mature Nuc forms, suggesting that SP_{Usp} impairs enzyme activity in vitro.

To test for SP_{Usp}:Nuc activity in vivo, whole-cell lysates were prepared on cultures producing SP_{Nuc}:NucB or SP_{Usp}:NucB and compared with a nonproducing strain. Whole-cell lysates were analyzed by agarose gel electrophoresis, and total genomic DNA was visualized by ethidium bromide staining. No DNA hydrolysis was detected in any sample (data not shown). We conclude from these results and in keeping with the good growth of strains producing SP_{Usp}:NucB that this precursor is active in vitro but not in vivo. This is consistent with the data of Poquet et al., who demonstrated that a mature form of Nuc produced in the cytoplasm is enzymatically active in the zymogram but inactive in vivo (42).

Altogether, these results suggest that the antifolding activity of SP_{Usp} is not better than that of SP_{Nuc} and thus suggest that the secretion enhancement is due to a better interaction between the precursor bearing the homologous SP_{Usp} and the host secretion chaperones (intermolecular feature).

Deletion of the natural propeptide severely reduces Nuc SE in *L. lactis*. The native Nuc propeptide is necessary for efficient secretion of Nuc driven by its native SP in *L. lactis* (32). The putative positive effects of the Nuc propeptide were also evaluated with the SP_{Usp} in place of the native SP_{Nuc}. A transcriptional and translational fusion between *usp45* expression and secretion signals and a fragment encoding NucT (devoid of its natural propeptide [32]) was constructed to produce SP_{Usp}:NucT (encoded by pUNuc4) (Table 1 and Fig. 1). SP_{Usp}:NucB (pUNuc1) and SP_{Usp}:NucT (pUNuc4) secretion levels were compared by Western blotting on protein samples prepared on exponential cultures of the corresponding *L. lactis* strains (Fig. 5). The total amounts of detected Nuc forms are comparable for the two strains. However, SE of SP_{Usp}:NucT is only 30%,

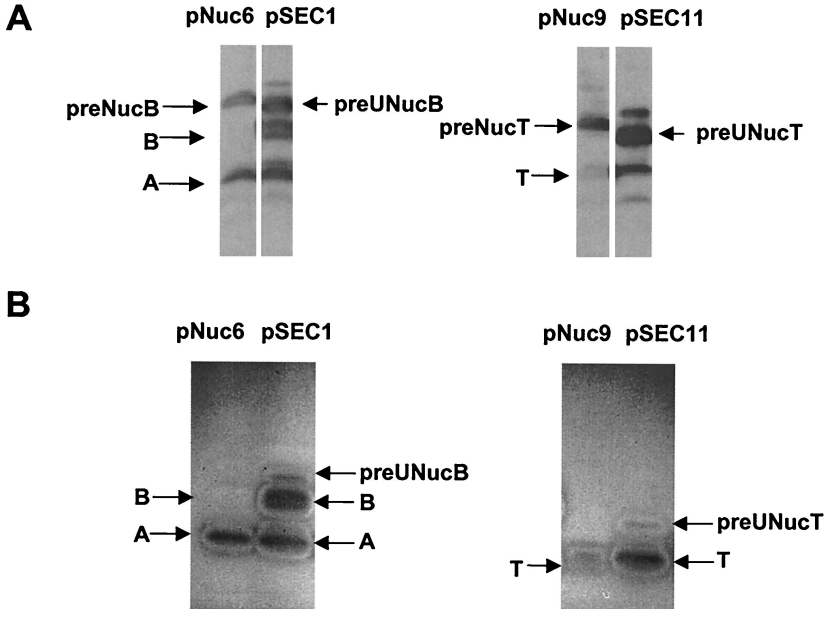

FIG. 4. Detection of enzymatic Nuc activity in precursor depends on the nature of the SP used to drive Nuc secretion. Protein samples were prepared from overnight cultures of lactococcal strains containing pNuc6 or pNuc9 (carrying cassette $P_{59}SP_{Nuc}$:NucB or $P_{59}SP_{Nuc}$:NucT, respectively) or nisin-induced cultures of lactococcal strains containing pSEC1 or pSEC11 (carrying cassette $P_{nisA}SP_{Usp}$:NucB or $P_{nisA}SP_{Usp}$:NucT, respectively). Strains containing pNuc6 and pNuc9 accumulate precursor forms in cell fraction. To achieve such accumulation with SP_{Usp}, strains containing pSEC1 and pSEC11 were strongly induced with 10 ng of nisin/ml for 1 h. (A) Western blot analysis of cell fractions of lactococcal strains producing Nuc. A faint band is visible upon the precursor band for pSEC1 or pSEC11. This band corresponds probably to precursor aggregation due to overproduction of SP_{Usp}:NucB and SP_{Usp}:NucT. (B) Zymogram for detection of enzyme activity performed with the same protein samples after SDS-PAGE and gel renaturation. The positions of SP_{Usp}:NucB (preUNucB), SP_{Usp}:NucT (preUNucT), SP_{Nuc}:NucB (preNucB), and SP_{Nuc}:NucT (preNucT) precursor forms and of NucB (B), NucT (T), and NucA (A) mature forms are indicated by arrows.

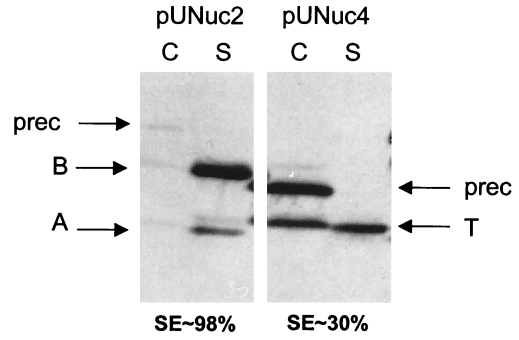

FIG. 5. Deletion of the natural propeptide strongly decreases Nuc SE. Nuc SE was estimated by Western blot analysis on exponential-phase cultures of lactococcal strains containing pUNuc1 (encoding SP_{Usp}:NucB) and pUNuc4 (encoding SP_{Usp}:NucT). Migration positions of precursor forms (prec) or mature forms of NucA (A), NucB (B), and NucT (T) are indicated by arrows. C, cell lysates; S, supernatant fraction; SE, the proportion of total protein which is present in mature secreted form.

compared to around 95% for SP_{Usp}:NucB. Some mature NucT is also found associated with the cell fraction; this was previously observed for native NucA and/or NucT forms in *L. lactis* and *C. glutamicum* (12, 32, 34). It could be due to electrostatic interactions between negatively charged cell wall and charged residues in the N terminus of NucA and NucT (12). These results confirm that the Nuc propeptide is needed for efficient Nuc secretion in *L. lactis* and that this effect is independent of the SP used.

The synthetic propeptide LEISSTCDA improves both the SE and yields of NucB and NucT secreted via SP_{Usp}. The synthetic propeptide LEISSTCDA exerts a positive effect when acting in combination with the native SP_{Nuc} (32). To test its effects when combined with a nonnative SP, we constructed fusions SP_{Usp}:NucT (encoded by pUNuc4) and SP_{Usp}:LEISSTCDA-NucT (encoded by pUNuc5) (Table 1 and Fig. 1) and compared secretion profiles by Western blotting (Fig. 6). SP_{Usp}:LEISSTCDA-NucT processing was significantly more efficient (above 95%) than that of SP_{Usp}:NucT (30%). In addition, SP_{Usp}:LEISSTCDA-NucT also displayed a three- to fourfold-greater overall Nuc yield than did SP_{Usp}:NucT (Fig. 6).

These results suggest that the LEISSTCDA propeptide may positively affect protein yield and/or the SE. To separate these potential effects, we examined the effects of LEISSTCDA propeptide on a protein that has a high SE, SP_{Usp}:NucB. Nuc secretion was analyzed in *L. lactis* strains producing SP_{Usp}:NucB (from pUNuc1) and SP_{Usp}:LEISSTCDA-NucB (from pUNuc3) (Fig. 6). As shown above, the SE of SP_{Usp}:NucB is already optimal (above 95%). Nevertheless, LEISSTCDA confers a significant increase (three- to fourfold) in the total Nuc yield. Analysis of plasmid DNA and Northern blotting confirmed that this increase was not due to differences in plasmid copy number or amounts of mRNA (data not shown). Furthermore, similar increases of detected Nuc forms were also observed when SP_{Usp}:LEISSTCDA-Nuc fusions were expressed from other promoters (data not shown). These results show that the LEISSTCDA synthetic propeptide may have positive effects on both SE (e.g., for SP_{Usp}:LEISSTCDA-NucT) and yield (e.g., for SP_{Usp}:LEISSTCDA-NucB).

The combination of these results shows that the enhancing effect of the synthetic propeptide on SE and yield does not depend on the nature of the SP used to direct Nuc secretion. Similar results were obtained when the LEISSTCDA propeptide preceded other heterologous secreted proteins (L. A. Ribeiro, V. Azevedo, Y. Le Loir, S. C. Oliveira, Y. Dieye, J. C. Piard, A. Gruss, and P. Langella, submitted for publication).

The SE of Nuc in *L. lactis* depends on the net global charge of the N terminus of the mature moiety. LEISSTCDA is characterized by its net global negative charge (−2), conferred by acidic amino acid residues at positions +2 and +8. To test whether these two acidic residues are necessary for secretion enhancement by LEISSTCDA, two new propeptides were designed such that acidic amino acids were replaced by neutral or basic residues: LGISSTCNA (neutral global net charge) and LKISSTCHA (positive global net charge of +2). A third propeptide, LQVDDIPSA, was also obtained; it has a different primary structure but contains two acidic residues at positions +4 and +5 (see Materials and Methods) (Table 2 and Fig. 7). The effects of the different propeptides were evaluated on SP_{Nuc}:NucB. *L. lactis* MG1363 strains secreting native NucB and LEISSTCDA-NucB were used as controls. Exponential-phase *L. lactis* cultures containing the different Nuc fusions were processed and examined by Western blotting. In each case, the three expected Nuc forms (precursor and mature NucB and NucA) were detected (Fig. 7). The presence of basic

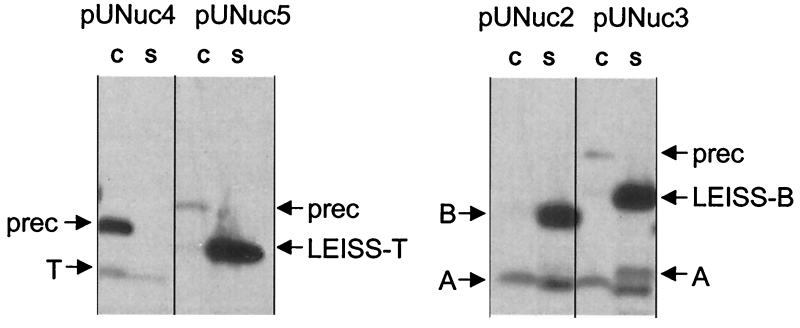

FIG. 6. The synthetic propeptide enhances Nuc SE when used in combination with SP_{Usp}. Nuc SE was estimated by Western blot analysis on exponential-phase cultures of four lactococcal Nuc-producing strains. Right panel, MG1363 containing pUNuc4 (encoding SP_{Usp}:NucT) and pUNuc5 (encoding SP_{Usp}:LEISSTCDA:NucT). Left panel, MG1363 containing pUNuc1 (encoding SP_{Usp}:NucB) and pUNuc3 (encoding SP_{Usp}:LEISSTCDA:NucB). Migration positions of precursor forms (prec) or mature forms of NucA (A), NucB (B), NucT (T), LEISSTCDA:NucT (LEISS-T), and LEISSTCDA:NucB (LEISS-B) are indicated by arrows. C, cell lysates; S, supernatant fraction.

TABLE 2. Oligonucleotides used in this study

Oligonucleotide no.	Sequence[a]	Description	Reference
1	5′ CTC GGA ATA TCG TCG ACT TGT AAT GCA 3′ L G I S S T C N A	Neutral propeptide coding strand	This work
2	5′ TT ACA AGT CGA TAT TCC GAG TGC A 3′	Neutral propeptide complementary strand	This work
3	5′ CTC AAA ATA TCG TCG ACT TGT CAT GCA 3′ L K I S S T C H A	Basic propeptide coding strand	This work
4	5′ TG ACA AGT CGA CGA TAT TTT GAG TGC A 3′	Basic propeptide complementary strand	This work
5	5′ TCT AGA GCG CCT ACA CTT TTG CTC 3′	P_{usp}:SP_{usp} amplification, forward primer	This work
6	5′ GTA TGC ATA A AAC ACC TGA CAA CGG GG 3′ *Nsi*I	P_{usp}:SP_{usp} amplification, reverse primer	This work
7	5′ GTCTAGAACCGAACTTAATGGGAG 3′	SP_{usp}:*nuc* amplification, forward primer	This work
8	5′ GGAATTCCGATCTAAAAATTATAAAAGT 3′	SP_{usp}:*nuc* amplification, reverse primer	31

[a] The sequence shown below the oligonucleotide sequence is the peptide sequence.

residues (on LKISSTCHA) drastically reduced Nuc SE, to around 40% (Fig. 7, lanes 11 and 12), compared to 70% obtained with native Nuc (Fig. 7, lanes 3 and 4) and 90% obtained with SP_{Nuc}:LEISSTCDA-Nuc (Fig. 7, lanes 5 and 6). The LKISSTCHA-Nuc fusion was found mainly in precursor form. This effect is consistent with our previous finding that NucT (containing three positive charges in the first 10 residues of the mature moiety) has an SE of 30% (32). In contrast, introduction of two different negatively charged propeptides (Fig. 7, lanes 5, 6, 7, and 8) leads to a higher SE and an increased yield compared to that of native Nuc (Fig. 7, lanes 3 and 4). Insertion of a neutral propeptide also increases Nuc SE (Fig. 7, lanes 7 and 8) as well as the quantity of secreted Nuc. A maximum increase in secreted Nuc yield was approximately fourfold compared to the control (Fig. 7, lanes 3 and 4). These results show that (i) a mature protein with a positively charged N-terminal end is poorly secreted in *L. lactis* and (ii) both negatively charged and neutral propeptides enhance Nuc secretion in *L. lactis* and exhibit a dual effect of improving SE and increasing protein yield. Taken together, these findings suggest that a synthetic propeptide (devoid of basic residues) may act as a spacer that separates the globular Nuc protein from the region involved in Nuc maturation by the signal peptidase and thus facilitates precursor processing in *L. lactis*.

DISCUSSION

SP effects on Nuc secretion. SPs, although poorly conserved in their primary structure, are characterized by a conserved tripartite secondary structure (61). In comparison, SP_{Nuc} has an atypical structure (37). It is 60 residues long (the mean size of gram-positive bacterial SP is 28 residues [57]) and contains two highly hydrophobic stretches of approximately the same length separated by a hydrophilic region containing three basic residues. Miller et al. (37) proposed a model for insertion of native SP_{Nuc}:Nuc precursor in the bacterial membrane where SP_{Nuc} forms a hairpin. This atypical structure may be poorly recognized by the lactococcal secretion machinery, thereby explaining why Nuc precursor accumulates in *L. lactis*.

The Usp45 signal peptide (SP_{Usp}) has a more consensual structure. The N-terminal region of Usp45 (including SP_{Usp} and, in some cases, several amino acids of the mature protein) has already been used to drive secretion of heterologous proteins in *L. lactis*, e.g., α-amylase (59), bovine plasmin (3), IL-2 and IL-6 (54), Nuc (12, 42), BLG (6a), NSP4 (13a), and lipase (13). When estimated, SE of these different fusions was heterogeneous. For instance, α-amylase fusion to SP_{Usp} resulted in 80% of precursor accumulation in the cell fraction (59). Precursor accumulation was also observed for lipase fusions

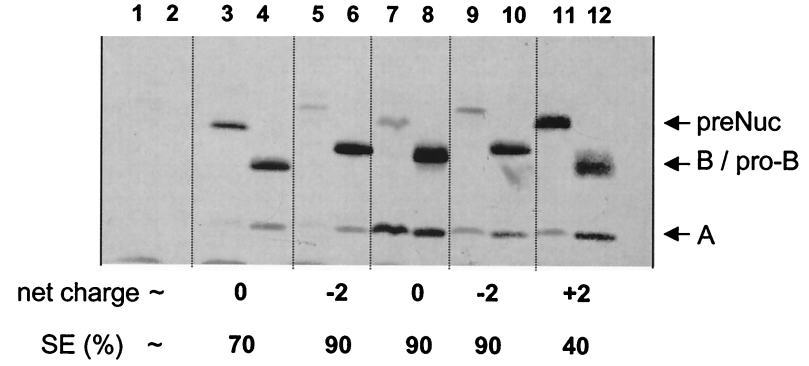

FIG. 7. Secretion profiles of Nuc with or without synthetic propeptide derivatives. Nuc SE was estimated by Western blot analysis on cell and supernatant fractions extracted from exponential-phase *L. lactis* cultures. Supernatant and cell fractions were prepared separately, and immunodetection of the different Nuc forms was performed after SDS-PAGE. SE and the net charge of the first 10 residues of the mature moiety (net charge) are given below each lane. Strains contain the cloning vector alone (lanes 1 and 2); pNuc13 encoding SP_{Nuc}:Nuc (lanes 3 and 4); pNuc14 encoding the fusion protein containing the original synthetic propeptide SP_{Nuc}:LEISSTCDA-Nuc (lanes 5 and 6); and the fusion proteins containing the mutated propeptides SP_{Nuc}:LGISSTCNA-Nuc (lanes 7 and 8), SP_{Nuc}:LQVDDIPSA-Nuc (lanes 9 and 10), and SP_{Nuc}:LKISSTCHA-Nuc (lanes 11 and 12). We noted heterogeneity in the apparent sizes of precursors and secreted proproteins on SDS-PAGE, possibly due to charge modifications introduced on the synthetic propeptides. preNuc, native Nuc precursor form; B, NucB; A, NucA; pro-B, mature forms of propeptides fused to NucB.

(13), whereas fusion of Nuc to SP_{Usp} plus the first 16 residues of the Usp45 mature moiety resulted in a good SE (12, 42).

We compared here the Nuc SE driven by its native SP with the Nuc SE driven by the homologous SP_{Usp}. Western blotting and pulse-chase experiments revealed a significant increase of Nuc SE when SP_{Usp} was used. Altogether, these results show that the use of a homologous SP may be necessary, but not sufficient, to guarantee efficient protein secretion. When homologous SP does not improve the SE of a given protein, some alternative tools such as synthetic propeptides may be useful as mentioned below.

How does the homologous SP_{Usp} enhance Nuc SE? The SP reportedly acts as an intramolecular chaperone to retard protein folding. The SP thus facilitates interactions with chaperones dedicated to secretion and participates in maintaining the precursor in a conformation compatible with translocation (14, 45, 57). In vitro studies demonstrate that interaction between SP and the mature protein moiety can greatly retard the kinetics of protein folding (35). Nevertheless, in the absence of an external chaperone, although folding is retarded, it often occurs, resulting in precursor activity (as shown elsewhere for several enzymes [6, 15, 23, 53]). However, in vivo, cytoplasmic activity of a secreted protein could be lethal for the cell; precursor activity may be prevented through interactions with the secretion machinery or a dedicated inhibitor (1, 7, 21). Here, we confirmed that SP_{Nuc}:Nuc precursors are inactive in vitro (32), suggesting a strong intramolecular chaperone activity for SP_{Nuc}. In contrast, SP_{Usp}:Nuc precursors have some enzymatic activity in zymogram tests. This result shows that the two SPs have different antifolding capacities. The lower antifolding capacity of SP_{Usp} suggests that its intramolecular chaperone activity is not better than that of SP_{Nuc}. SP_{Usp} may then improve Nuc SE by allowing a better recognition of SP_{Usp}:Nuc precursor by the lactococcal secretion machinery (intermolecular interaction).

Effects of native and synthetic propeptides on SE of Nuc. Long propeptides that are present, for example, in proproteases have intramolecular chaperone activities and are involved in protein folding, protein secretion, and inhibition of enzyme activity (52). However, little is known about the role of short propeptides (e.g., those present in barnase, some amylases, and Nuc). Our studies rule out a role of Nuc propeptide in Nuc enzymatic activity, in keeping with previous reports (9, 32). In *L. lactis*, we observed a positive effect of the natural Nuc propeptide that is independent of the SP that precedes it. A synthetic propeptide, LEISSTCDA, was previously described as a secretion enhancer that can mimic the positive role of native Nuc propeptide in the SE and yield of both NucB and NucT (32).

In addition to LEISSTCDA propeptide, LQVDDIPSA and LGISSTCNA have similar effects in improving NucB secretion. All these peptides are devoid of basic residues. These results suggest that acidic and neutral residues are equally efficient in enhancing Nuc secretion in *L. lactis*. It is notable that other SP_{Usp} fusions that include an N terminus having a global net charge of −2 also appeared to be efficiently secreted (12, 42). In contrast, a fusion to the basic propeptide LKISSTCHA is very poorly secreted in *L. lactis*. The behavior of these fusions suggests that, at least for the gram-positive *L. lactis*, proteins designed for membrane translocation have similar charge requirements as in *E. coli*. In both cases, basic residues at the mature N terminus may drastically reduce SE (33, 36), while the presence of an acidic or neutral spacer improves SE.

Effect of synthetic propeptide on protein yield. The combination of the host SP_{Usp} with synthetic propeptide LEISSTCDA led to protein yields slightly higher (around 25 mg/liter) than those observed with pNuc7 (described in reference 32, combining SP_{Nuc} and LEISSTCDA). Possibly, in the absence of any synthetic propeptide, the precursor SP_{Usp}:Nuc is subject to a partial intracellular degradation; in this case, the real SE would actually be lower than the apparent SE. In the case of NucB, this may result in an apparent optimal SE. The insertion of a synthetic propeptide could affect the charge balance in the area of the SP cleavage site and/or the conformation of the precursor. The resulting effect could render the precursor less sensitive to intracellular degradation and/or could help it to escape the intracellular degradation thanks to a better SE. Degradation of hybrid precursor has already been observed in *B. subtilis* due to a poor SE of this precursor (38). Hybrid precursor could be a target for degradation by intracellular or membrane proteases such as ClpP (16) or HtrA (43). We are currently addressing this question by comparing yields and SEs of protein fusions in the different mutant backgrounds.

Combination of homologous SP and synthetic propeptide for the design of new secretion tools. The SP_{Usp}:LEISSTCDA combination can be used to direct secretion of other heterologous proteins in *L. lactis* (Ribeiro et al., submitted). In some cases, protein production is increased when LEISSTCDA propeptide is inserted between SP_{Usp} and the mature moiety of the hybrid precursor (Ribeiro et al., submitted). In those studies, the synthetic propeptide insertion did not interfere with antigenic properties or with activity of the heterologous protein. These results indicate that the synthetic propeptide can improve secretion of heterologous proteins other than Nuc. Recently, we successfully used, in *L. lactis*, the combination of SP_{Usp} and LEISSTCDA to improve the secretion of L7/L12, the *Brucella abortus* immunodominant antigen (Ribeiro et al., submitted). The host range of this combination is being currently evaluated. We propose that the combination of SP_{Usp} and a properly designed synthetic propeptide such as the nonapeptides reported here could be a valuable tool for enhancement of secretion of heterologous proteins in gram-positive bacteria, including various LAB species such as *Streptococcus thermophilus, Lactobacillus casei*, and *Lactobacillus sakei*.

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