

Lactococcus lactis, an Efficient Cell Factory for Recombinant Protein Production and Secretion

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Key Words

Lactococcus lactis · Recombinant protein · Expression systems · Protein secretion · Production optimization

Abstract

The use of Gram-positive bacteria for heterologous protein production proves to be a useful choice due to easy protein secretion and purification. The lactic acid bacterium *Lactococcus lactis* emerges as an attractive alternative to the Gram-positive model *Bacillus subtilis*. Here, we review recent work on the expression and secretion systems available for heterologous protein secretion in *L. lactis*, including promoters, signal peptides and mutant host strains known to overcome some bottlenecks of the process. Among the tools developed in our laboratory, inactivation of HtrA, the unique housekeeping protease at the cell surface, or complementation of the Sec machinery with *B. subtilis* SecDF accessory protein each result in the increase in heterologous protein yield. Furthermore, our lactococcal expression/secretion system, using both P_{Zn} *zitR*, an expression cassette tightly controlled by environmental zinc, and a consensus signal peptide, SP_{Exp4}, allows efficient production and secretion of the staphylococcal nuclease, as evidenced by protein yields (protein amount/biomass) comparable to those obtained

using NICE or P170 expression systems under similar laboratory conditions. Finally, the toolbox we are developing should contribute to enlarge the use of *L. lactis* as a protein cell factory.

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Introduction

To date, many expression systems have been developed to produce recombinant proteins for various biotechnological applications. Among prokaryotic systems, the highest protein levels are obtained using *Escherichia coli* as the cell factory [Jana et al., 2005]. However, in *E. coli*, the most commonly used production strategies are intracellular (in the periplasm or even in the cytoplasm), and involve expensive and often problematic downstream purification processes. Moreover, endotoxin or lipopolysaccharide should be removed from proteins to be administered to humans.

In contrast, heterologous proteins produced in Gram-positive bacterial hosts can be easily secreted into the medium, thus facilitating their purification. *Bacillus* species have been widely used to produce enzymes, even though most of them are homologous and naturally-secreted

proteins, like alkaline proteases used in washing agents or amylases for the starch industry [for review, see Westers et al., 2004]. *Bacillus subtilis* is endotoxin free and considered as a 'generally recognized as safe' micro-organism [Li et al., 2004]. However, many heterologous proteins secreted by *B. subtilis* are degraded by its complex extracellular proteolytic system, comprising 7 secreted and 5 cell surface proteases [for review, see Westers et al., 2004]. Mutant strains defective for several extracellular proteases have been constructed; in particular, WB800 is devoid of all 7 secreted proteases and the cell surface protease WprA [Wu et al., 2002]. Extracellular proteolysis is thus only limited without being abolished, as HtrA and CtpA family members are still present.

Another Gram-positive bacterium having a 'generally recognized as safe' status, the lactic acid bacterium model *Lactococcus lactis*, is becoming an attractive alternative for heterologous protein secretion. *L. lactis* has been studied for the last 2 decades: its metabolism is relatively simple and well known, and the genome of the IL1403 laboratory strain is sequenced [Bolotin et al., 2001]. *L. lactis* presents similar putative bottlenecks in protein production and secretion as *B. subtilis* (fig. 1), but it also provides several advantages. Only 1 major protein, Usp45, is secreted into the medium (at a level detectable by Coomassie brilliant blue staining) [van Asseldonk et al., 1993], thus simplifying downstream purification processes. Laboratory strains possess only 1 exported housekeeping protease, HtrA, and an extracellular protease-free mutant is available [Poquet et al., 2000, 2001]. Finally, although the ways to reach a high biomass level will not be discussed here [Riesenberg and Guthke, 1999], *L. lactis* growth under fermentation conditions allows an easy scale-up [Mierau et al., 2005a].

Here, we first review the tools that have been developed and used to produce and secrete heterologous proteins in *L. lactis* before describing our recent advances in putting together an innovative toolbox.

Gene Expression Systems

A lot of expression systems have been described in *L. lactis* [for reviews, see de Vos, 1997, 1999]. In the following section, we focus on either constitutive or inducible promoters that have been used for heterologous gene expression in *L. lactis*.

Constitutive Promoters

In *L. lactis*, numerous identified promoters are not known to be controlled by any regulator or growth conditions, and are presumed to be constitutive under labo-

ratory growth conditions. The most commonly used promoters were randomly isolated from an *L. lactis* genomic library as fusions to the promoterless *cat-86* reporter gene conferring chloramphenicol resistance [van der Vossen et al., 1987]. Five promoters – P21, P23, P32, P44 and P59 – were obtained as active transcriptional fusions in both *L. lactis* and *B. subtilis*. Strong (P21, P23 and P59) and weak (P32 and P44) promoters were distinguished by the chloramphenicol acetyl transferase activity levels, and the sequences of the strongest promoters were found to best fit with the consensus [van der Vossen et al., 1987]. These promoters have been used to produce numerous heterologous proteins in *L. lactis* (table 1).

Inducible Promoters

Inducible promoters generally drive the expression of genes involved in cell adaptation to its environment. Many *L. lactis* promoters are known to be inducible either by stress conditions such as phage attack, temperature or pH shift, or by a specific sugar [for reviews, see de Vos, 1997, 1999; for a recent example, see Miyoshi et al., 2004]. Regulated promoters are of particular interest in the case of putatively toxic genes whose repression should allow biomass increase prior to induction at a defined culture phase/time to produce the desired proteins.

The nisin-inducible controlled gene expression (NICE) system [de Ruyter et al., 1996; for reviews, see Kuipers et al., 1995, 1997, 1998] derives from the *nis* (*nisABTCIPRKEFG*) operon present in some *L. lactis* strains, which is involved in the biosynthesis of the antimicrobial peptide nisin [Kuipers et al., 1998]. NICE comprises the regulatory elements of the *nis* operon: P_{nisA} , the nisin-inducible promoter (cloned into several expression vectors), and *nisRK*, the regulator-sensor 2-component system (either carried by compatible plasmids or integrated in the chromosome). The NICE system has been extensively used to produce proteins in *L. lactis* [for reviews, see Kunji et al., 2003; Le Loir et al., 2005; Mierau and Kleerebezem, 2005]. It offers numerous advantages: (1) easy use, (2) tightly controlled and efficiently induced expression leading to high protein yields [Mierau and Kleerebezem, 2005], and (3) large-scale production process, as recently shown for *Staphylococcus simulans* lysostaphin [Mierau et al., 2005a, b]. Growth conditions, fermentation parameters and nisin amounts have been optimized, leading to a lysostaphin yield increase from 100 to 300 mg/l [Mierau et al., 2005b]. The process is equally effective for production scales of 1, 300 or even 3,000 liters, without any loss of productivity [Mierau et al., 2005a, b]. However, for industrial production, nisin addition remains costly and, in the

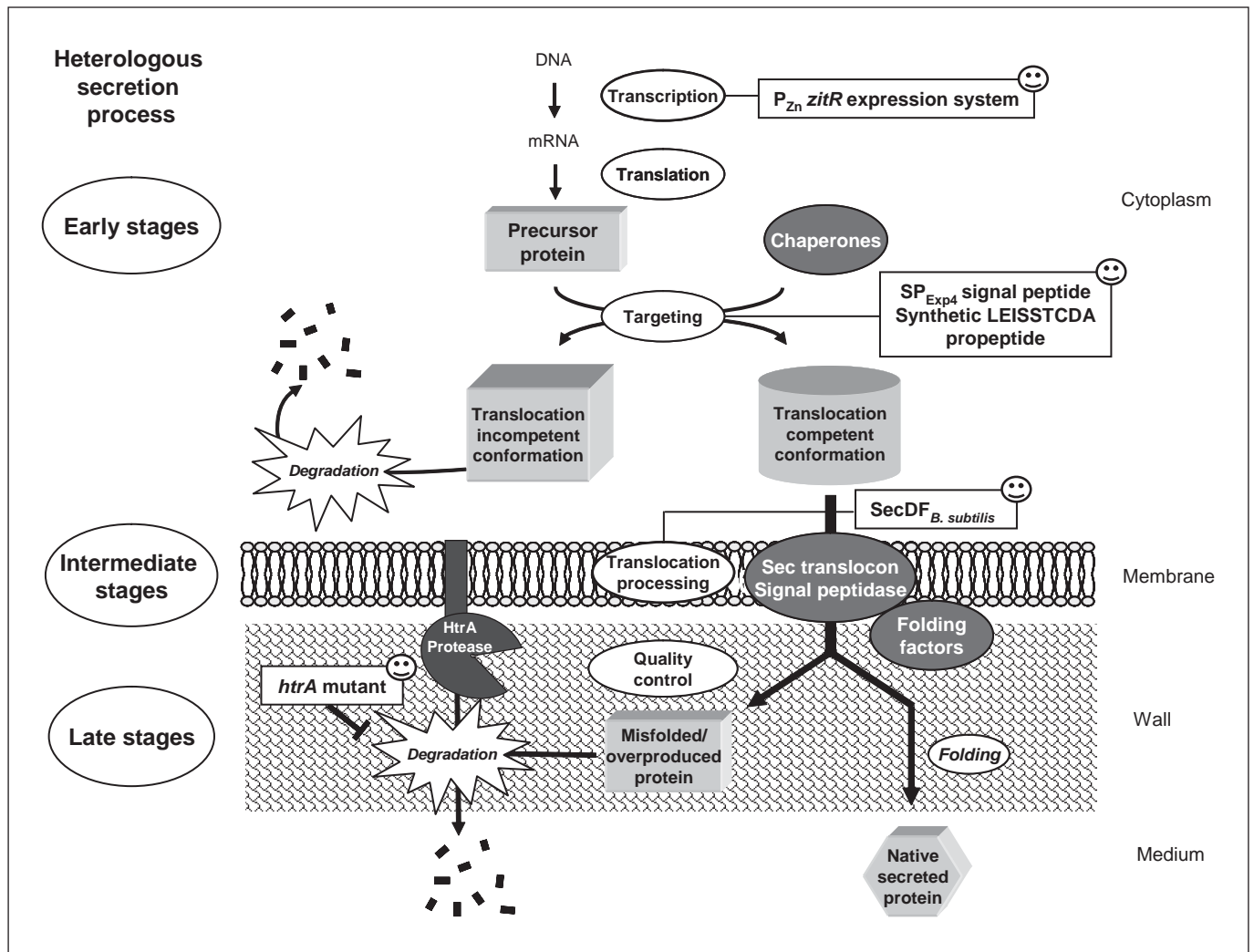


Fig. 1. Heterologous protein secretion in *L. lactis*. In *L. lactis*, as in *B. subtilis*, the heterologous secretion [for reviews, see Li et al., 2004 and Westers et al., 2004] can be divided into 3 stages: (1) early secretion steps in the cytoplasm, which include precursor synthesis and targeting to the translocation machinery, (2) intermediate secretion steps that allow translocation of the precursor

across the membrane (through the Sec translocon) and signal peptide cleavage, and (3) late secretion stages corresponding to protein quality control, which comprise the degradation of overproduced or misfolded proteins and the folding of mature protein and its release into the medium. The tools reported here are marked by the following icon: ☺.

particular case of a protein of pharmaceutical interest, a downstream removal process should be required.

The P170 gene expression system [Israelsen et al., 1995; Madsen et al., 1999] has also been reported to be compatible with industrial production and fermentation processes in *L. lactis*. P170, the natural promoter of an uncharacterized gene termed *orfX*, is induced by the pH decrease (pH < 6) during transition from postexponential to stationary phases of glucose-grown cultures [Bredmose et al., 2001; Madsen et al., 1999]. P170 induction depends

on RcfB, a positive regulator of the CRP-FNR family which binds on ACiD-boxes [Madsen et al., 2005]. For protein production, P170 offers the major advantage (compared to the NICE system) of self-inducibility via lactic acid accumulation in the medium during growth. Using P170, the staphylococcal nuclease (Nuc) yield reaches 300 mg/l. Scale-up experiments from 1 to 200 liters in 1 step have been achieved leading to similar protein yield, purity and stability (Bioneer; <http://www.lactococcus.dk/>).

Table 1. Heterologous proteins produced in *L. lactis* under the control of constitutive promoters

Protein	Origin	Promoter	Strain	Reference
Chitinase	<i>Serratia marcescens</i>	P32, P59	MG1363	Brurberg et al., 1994
M6	<i>Streptococcus pyogenes</i>	P23, P59	IL1403	Piard et al., 1997
SlpH	<i>Lactobacillus helveticus</i>	P32	MG1363	Callegari et al., 1998
Phenylalanine ammonia-lyase	<i>Petroselinum crispum</i>	P32	MG1363	Xiang et al., 1999
Carnobacteriocin A	<i>Carnobacterium piscicola</i>	P32	MG1363	Franz et al., 2000
Ply118, Ply511	<i>Listeria monocytogenes</i>	P21, P32, P59	MG1363	Gaeng et al., 2000
ClfA	<i>Staphylococcus aureus</i>	P23, P59	MG1363	Que et al., 2000
FnBPA	<i>S. aureus</i>	P23	MG1363	Que et al., 2001
Nuc	<i>S. aureus</i>	P59	MG1363	Dieye et al., 2001
VP2, VP3	Infectious bursal disease virus	P59	MG1363	Dieye et al., 2003
Cu/Zn superoxide dismutase	<i>Homo sapiens</i>	P32	MG5267	Wei et al., 2003
Enterocin A	<i>Enterococcus faecium</i>	P32	IL1403	Martinez et al., 2000
Pediocin PA-1	<i>Pediococcus acidilactici</i>			
Neutral protease	<i>B. subtilis</i>	P32	MG1363	van de Guchte et al., 1990
Lipase	<i>Staphylococcus hyicus</i>	P44	NZ9000	Drouault et al., 2000

A new expression system in *L. lactis*, P_{Zn} *zitR*, has recently been developed. It naturally controls expression of the *zit* operon (*zitRSQP*). By homology with streptococcal *adc* operons, *zit* is putatively involved in Zn^{2+} uptake by the ABC transporter ZitSQP, and it is controlled by ZitR, an MarR family transcriptional regulator [Llull and Poquet, 2004; Llull et al., in preparation]. An expression vector including P_{Zn} *zitR* promoter-regulator region, pVE8062, was constructed [Llull and Poquet, 2004]. Using 2 reporter proteins, a secreted recombinant Nuc (SP_{Usp45} -Nuc) or a cytoplasmic β -galactosidase, *Leuconostoc mesenteroides* LacLM, P_{Zn} *zitR*-controlled expression proved to be tightly regulated in response to zinc concentration in the medium [Llull and Poquet, 2004]. Furthermore, P_{Zn} *zitR* has been compared to NICE using the above-mentioned reporter genes (cloned in the same contexts), and expressed in each case under defined induction conditions (EDTA 30 μ M or nisin 1 ng/ml in chemically defined SA medium) [Jensen et al., 1993]. After 3 h of induction, P_{nisA} gave a 5-fold higher level of LacLM activity than P_{Zn} , but Western blotting showed a similar Nuc protein yield for both systems [Llull and Poquet, 2004].

We propose the following model for the regulation of either *zit* or any heterologous gene cloned under P_{Zn} *zitR* control [Llull and Poquet, 2004; Llull et al., in preparation]. Under zinc excess in the medium (in a large range of concentrations), ZitR bound to P_{Zn} represses expression by competing with RNA polymerase binding (fig. 2a). Alternatively, under extreme Zn^{2+} starvation that can be

achieved either through EDTA addition or growth in a zinc-poor medium (like SA medium), ZitR becomes inactive and allows RNA polymerase binding to P_{Zn} and transcription initiation (fig. 2b). The P_{Zn} *zitR* system thus appears well suited for heterologous expression control. If heterologous protein toxicity is a risk, zinc addition should allow repression and biomass increase before induction by EDTA at the late exponential growth phase (note that EDTA is both food grade and present in the suspension buffers of several injectable therapeutic products including proteins; <http://www.fda.gov/cder/biologics>). Alternatively, if moderate heterologous protein levels can be tolerated during the exponential phase, the use of a zinc-poor medium should allow a progressive self-induction without any additions [Llull and Poquet, 2004; Poquet and Llull, 2006]. This production process is of particular interest because it is cheap and compatible with large-scale production.

Protein Secretion in *L. lactis*

L. lactis is able to secrete proteins bearing an N-terminal signal peptide into the growth medium via the Sec pathway (fig. 1). Early secretion stages involve protein precursor recognition and targeting to the membrane translocation machinery, which are assumed to be co-translational via the dedicated chaperone signal recognition particle. During translocation, the precursor protein crosses the membrane through the Sec translocon. Late secretion steps include signal peptide cleavage by the leader peptidase, mature protein release and folding. In

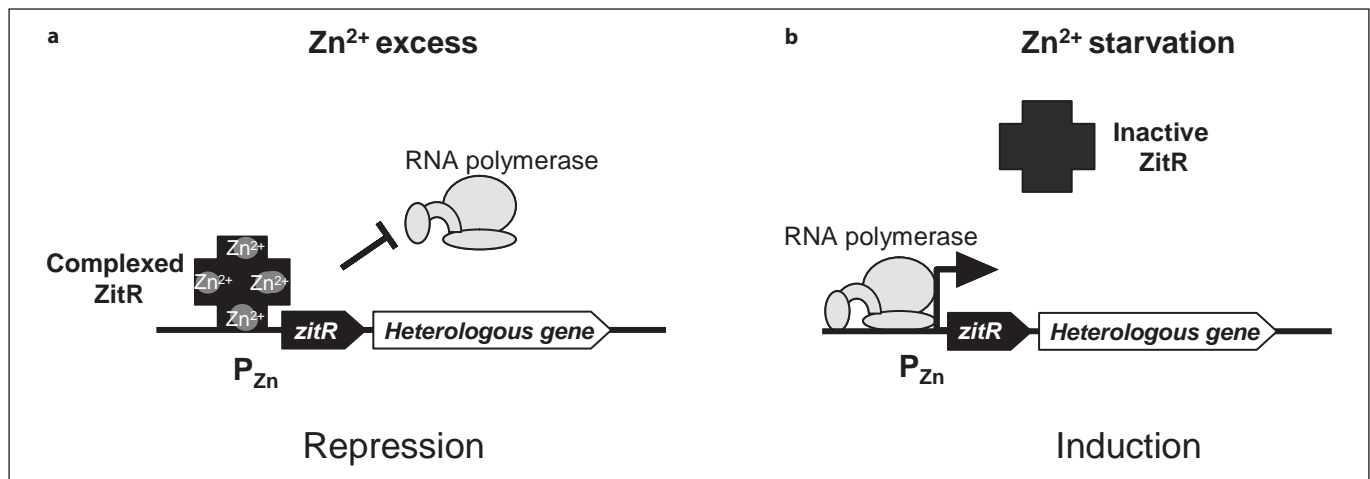


Fig. 2. Model for P_{Zn} *zitR* regulation of heterologous protein expression. Under extracellular zinc excess conditions (a), ZitR repressor binds P_{Zn} promoter region and prevents transcription initiation by RNA polymerase, whereas zinc starvation conditions (b), obtained either by EDTA chelator addition or by growth in zinc-poor medium, allow transcriptional switch-on and induced expression.

the following section, we present the export signals used to drive heterologous secretion in *L. lactis*, and *L. lactis* mutant strains that can improve this process.

Addressing Targeted Proteins to the Sec Pathway

L. lactis signal peptides have been identified and characterized either randomly or studying specific exported proteins. *L. lactis* naturally secretes only 1 major protein, Usp45, possibly involved in cell wall hydrolysis and cellular segregation (as deduced by homology) [van Asseldonk et al., 1993]. Usp45 signal peptide (SP_{Usp45}) is commonly used for heterologous protein secretion in *L. lactis*. SP_{Usp45} has been combined with either a constitutive promoter like P59 [Dieye et al., 2001] or the inducible NICE system [for review, see Bermudez-Humaran et al., 2003a, b; Le Loir et al., 2005; Mierau et al., 2005b; Novotny et al., 2005]. Other signal peptides were identified in screens as reporter fusions, either alone [Perez-Martinez et al., 1992] or as combined expression and secretion cassettes [Poquet et al., 1998; Ravn et al., 2000; Sibakov et al., 1991]. One of them, SP310, was optimized by site-directed mutagenesis [Ravn et al., 2003]. Our library of signal peptides [Poquet et al., 1998] included SP_{Exp4}, whose structure and amino acid composition best fit with the Gram-positive consensus [Poquet and Llull, 2006]. SP_{Exp4} proved to efficiently drive secretion of the murine IL-12 p40 subunit in *L. lactis* [Bermudez-Humaran et al., 2003b].

A short synthetic propeptide (LEISSTCDA) can be fused between a signal peptide and the mature part of the

protein to improve the efficiency of heterologous secretion in *L. lactis*, as shown for several proteins (Nuc, *Bruceella abortus* L7/L12 antigen or *Bacillus stearothermophilus* α -amylase) [Le Loir et al., 1998, 2001, 2005]. The LEISSTCDA effect was observed in different contexts, using different proteins and signal peptides (both SP_{Nuc} signal peptide [Le Loir et al., 1998] and lactococcal SP_{Usp45} [Le Loir et al., 2001] have been used in the case of Nuc). It is likely that the negatively charged residues of the propeptide optimize the charge balance around the transmembrane domain of the signal peptide and thus enhance its efficiency and protein translocation [Le Loir et al., 1998, 2001]. LEISSTCDA fusion leads to an overall increase in protein yield, suggesting an indirect effect on intracellular proteolysis of the precursor [Le Loir et al., 2005].

Host Factors Affecting Secretion and/or Production of Heterologous Proteins

Secretion in Gram-positive bacteria is a multistep process, and factors localized in all cell compartments might become limiting in the case of overproduced heterologous proteins (fig. 1). A secretion stress was described in *B. subtilis* as a result of overproduction of a heterologous secreted protein, leading in turn to a global regulatory response including genes involved in stress resistance and secretion [Hyyryläinen et al., 2005]. In *L. lactis*, secretion factors have essentially been identified by homology with *E. coli* and *B. subtilis*. Here, we describe studies aimed at improving heterologous secretion, essentially by overproducing

limiting factors or inactivating adverse ones. Random mutagenesis has also been performed for this purpose [Nouaille et al., 2004; Morello et al., submitted].

Intracellular Targeting Factors. Bacterial *ffh* genes encode the protein component of signal recognition particle and are involved in protein secretion and folding [Tjalsma et al., 2004]. In *B. subtilis*, *ffh* is induced as part of the response to a severe secretion stress [Hyyryläinen et al., 2005]. Our attempts to overproduce *L. lactis* *ffh* in strains producing an inefficiently secreted Nuc form failed to increase its yield [Morello et al., unpubl. data].

Translocation Machinery. *L. lactis* translocon includes (1) SecA, the ATPase-dependent motor, which partially provides the energy required for preprotein translocation, and (2) SecY, SecE and SecG integral membrane proteins, which form the conducting channel through the hydrophobic membrane environment. The lactococcal Sec machinery is simpler than those of *B. subtilis* or *E. coli*, as the SecDF accessory component (either both *E. coli* SecD and SecF proteins or *B. subtilis* SecDF Siamese twin protein) is missing. *B. subtilis* SecDF enhances protein translocation by an as yet unknown mechanism [Bolhuis et al., 1998 and references therein]. Its expression in *L. lactis* results in increased yield of an inefficiently secreted Nuc form and of a *B. abortus* L7/L12 antigen, possibly by an indirect stabilization effect during translocation [Nouaille et al., 2006].

Signal Peptide Cleavage. During or shortly after preprotein translocation across the membrane, the signal peptide is removed by type I signal peptidase, releasing the mature form. In *B. subtilis* or *Bacillus megaterium*, processing relies on several signal peptidases and represents a bottleneck for some heterologous secreted proteins [Bolhuis et al., 1999; Malten et al., 2005]. In *L. lactis*, there is a unique type I signal peptidase, SipL. SipL overproduction in an *L. lactis* strain producing the inefficiently secreted Nuc form did not improve its secretion [Morello et al., unpubl. data].

Surface Quality Control Proteins. Proteins that are transported through the Sec machinery are unfolded. When they emerge from the membrane, proper folding into their native conformation is ensured by a protein quality control network involving (1) folding factors like chaperones and folding catalysts, and (2) housekeeping proteases to degrade misfolded/unfolded proteins [for Gram-positive bacteria, see review by Sarvas et al., 2004]. *L. lactis* quality control factors affect heterologous protein secretion.

L. lactis PmpA is a lipoprotein belonging to the peptidyl-prolyl-*cis/trans*-isomerase family. This protein is ho-

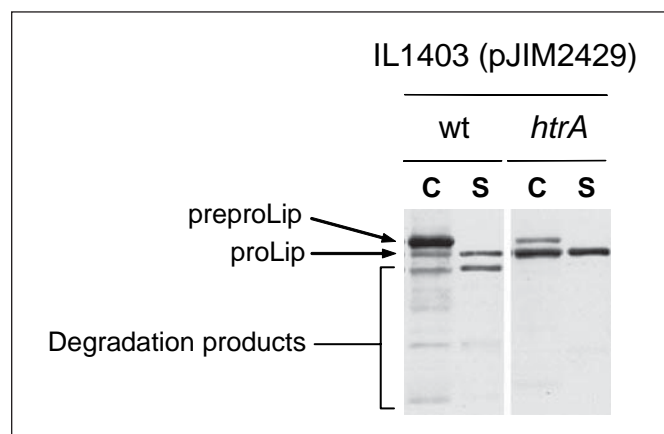


Fig. 3. *S. hyicus*-secreted lipase (Lip) is stable in an *L. lactis* *htrA* mutant strain. Lip secretion is compared in IL1403 wild-type (wt) and *htrA* mutant strains carrying the pJIM2429 expression vector (P23 *lip*) [Drouault et al., 2000]. IL1403 (pJIM2429) and IL1403*htrA* (pJIM2429) strains were grown exponentially at 30°C and proteins from cells (C) or supernatant (S) fractions were analyzed after SDS-PAGE by Western blotting using antibodies against the Lip C-terminus. Precursor (preproLip) and mature form (proLip) after signal peptide processing and degradation products are shown.

mologous to *B. subtilis* PrsA, a general surface folding factor whose overproduction is known to improve heterologous protein secretion [Kontinen et al., 1991, 1993]. *L. lactis* PmpA has been proposed to be a surface chaperone [Drouault et al., 2002]. When Lip, the secreted lipase of *Staphylococcus hyicus*, is produced in *L. lactis*, it is highly degraded, but this degradation can be inhibited by PmpA overproduction [Drouault et al., 2002]. This study showed that extracellular folding of heterologous proteins like Lip can be inefficient in *L. lactis*, and that PmpA overproduction can overcome this problem.

In *L. lactis*, HtrA is the unique exported housekeeping protease involved in protein quality control at the cell surface [Poquet et al., 2000]. HtrA is responsible for clearing abnormal proteins, such as reporter fusions, from the surface, and is both essential and induced under several stress conditions [Foucaud-Scheunemann and Poquet, 2003; Poquet et al., 2000]. *htrA* inactivation abolished the degradation of heterologous exported proteins, like Lip, allowing a 4-fold yield increase (fig. 3) [Miyoshi et al., 2002; Poquet et al., 2000, 2006]. All tested proteins are entirely stable in *htrA* mutant, suggesting that this mutant is protease-free at the cell surface [Poquet et al., 2000, 2001, 2006], a major advantage for developing *L. lactis* as a cell factory for protein production and secretion.

Table 2. Toolbox for protein production and secretion in *L. lactis*

Name	Characteristics	Reference
<i>Lactococcal mutant strains</i>		
IL1403 <i>htrA</i>	<i>htrA</i> mutant of IL1403 strain <i>htrA</i> inactivated by single crossing-over recombination, Cm ^R	Poquet et al., 2000
NZ9000 <i>htrA</i>	<i>htrA</i> mutant of NZ9000 (MG1363:: <i>nisRK</i>) strain <i>htrA</i> inactivated by single crossing-over recombination, Erm ^R	Miyoshi et al., 2002
NZ9000 Δ <i>htrA</i>	<i>htrA</i> mutant of NZ9000 (MG1363:: <i>nisRK</i>) strain <i>htrA</i> inactivated by internal deletion (double crossing-over recombination)	Rigoulay et al., 2005; Cortes-Perez et al., 2006
MG1363 Δ <i>htrA</i>	<i>htrA</i> mutant of MG1363 <i>htrA</i> inactivated by internal deletion (by the same strategy used for NZ9000 Δ <i>htrA</i>)	Madsen et al., unpubl. data
NZ9000 SecDF	NZ9000 (pSecDFI)	Nouaille et al., 2006
<i>Plasmids</i>		
pSecDFI	pAM β 1/ColE1; Erm ^R , Amp ^R ; <i>secDF</i> _{B. subtilis} encoding wild-type <i>B. subtilis</i> SecDF protein	Nouaille et al., 2006
pVE8064	pAM β 1/ColE1; Erm ^R , Amp ^R ; fusion between lactococcal <i>usp45</i> and staphylococcal <i>nuc</i> genes encoding SP _{Usp45} -NucB and expressed under P _{Zn} <i>zitR</i> control	Llull and Poquet, 2004
pLB145	pWV01; Cm ^R ; expression/secretion vector; fusion between lactococcal <i>exp4</i> and staphylococcal <i>nuc</i> genes encoding SP _{Exp4} -NucB and expressed under P _{Zn} <i>zitR</i> control	this work

A New Expression/Secretion System for Heterologous Proteins in *L. lactis*

To improve heterologous protein secretion in *L. lactis* (fig. 1), we are developing an innovative toolbox comprising a set of mutant strains and an integrated expression and secretion system (table 2).

Strains

The *L. lactis htrA* mutant strain proved to efficiently stabilize heterologous secreted proteins [Miyoshi et al., 2002; Poquet et al., 2000, 2006]. Several different *htrA* mutant strains are available and have been constructed in different genetic backgrounds and by different strategies (table 2).

An alternative strategy to improve the secretion process is to overproduce an enhancing factor. A genome comparison approach led to the complementation of *L. lactis* Sec translocon by *B. subtilis* SecDF (cloned in pSecDF1 plasmid; table 2), leading to an increase in protein yield [Nouaille et al., 2006].

Towards a New Expression/Secretion System

A new expression and secretion system was recently developed by combining the P_{Zn} *zitR* expression system, the SP_{Exp4} signal peptide and the *htrA* mutant strain.

P_{Zn} zitR-Driven Heterologous Expression. Efficiency of the P_{Zn} *zitR* expression system was evaluated in the *htrA* mutant strain context, using a recombinant Nuc form

(SP_{Usp45}-Nuc) as a heterologous reporter. Northern blotting (fig. 4) of extracts from the MG1363 Δ *htrA* (pVE8064) strain (table 2) grown in SA medium shows a basal expression level of the reporter during early exponential growth (OD₆₀₀ = 0.1 or T₀; fig. 4). Levels increase in the course of growth (data not shown) as a result of P_{Zn} self-induction in SA medium [Llull and Poquet, 2004; Poquet and Llull, 2006]. Successful induction or repression can be achieved in an *htrA* mutant strain under the same conditions as in a wild-type strain [Llull and Poquet, 2004]: addition at T₀ of 30 μ M EDTA or 10 μ M ZnSO₄ leads to an increase in expression greater than 150-fold, or to a complete expression switch-off after either 0.5 or 1 h of the respective treatments (fig. 4b). These results show that the tight control of P_{Zn} *zitR*-driven expression is functional in an *htrA* strain context.

SP_{Exp4}-Driven Heterologous Secretion. SP_{Exp4} efficiency for heterologous secretion was compared to that of SP_{Usp45} as a reference, using Nuc as a reporter (SP_{Exp4}-Nuc and SP_{Usp45}-Nuc recombinant proteins were cloned into the same expression vector; fig. 5a). Western blot analysis reveals the same production and secretion profiles, no matter what signal peptide was used (fig. 5b). This result shows that SP_{Exp4} and SP_{Usp45} are equally effective for Nuc secretion. SP_{Exp4} thus represents an interesting alternative for efficient heterologous protein secretion in *L. lactis*.

Fig. 4. Efficiency of P_{Zn} *zitR* expression system in an *L. lactis htrA* mutant strain. **a** Heterologous expression and secretion cassette from pVE8064 [Lull and Poquet, 2004]. In pVE8064, a recombinant Nuc form, SP_{Usp45} -Nuc (encoded by *usp_{SP-nuc}* fusion) is produced under P_{Zn} *zitR* control. **b** Northern blot analysis. MG1363 $\Delta htrA$ (pVE8064) strain was grown in chemically defined SA medium at 30°C until $OD_{600} = 0.1$ (T_0). At T_0 , cultures were divided into 2 subcultures. $ZnSO_4$ or EDTA was added at final concentrations of 10 or 30 μM , respectively. Sample cultures were taken at $T_0 + 0.5$ h or $T_0 + 1$ h; after RNA extraction, *usp_{SP-nuc}* mRNA was revealed by a ^{32}P -labelled *nuc* probe.

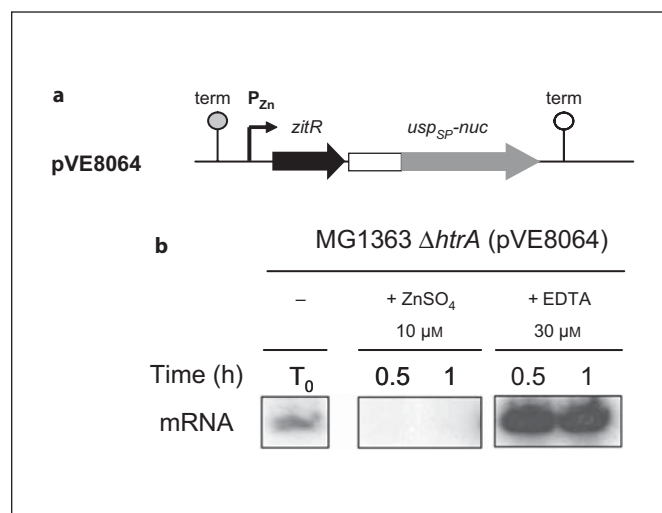
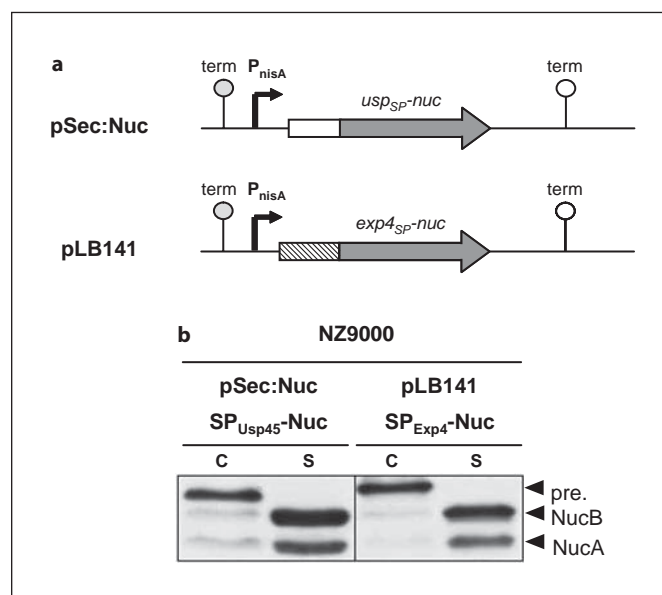


Fig. 5. Secretion efficiency of SP_{Exp4} compared to that of SP_{Usp45} . **a** Heterologous expression and secretion cassettes of pSec:Nuc and pLB141. In pSec:Nuc (also called pSEC1) [Le Loir et al., 1998] and pLB141 (constructed from pSec:p35-p40 [Bermudez-Humaran et al., 2003b], and thus containing the same backbone vector as pSec:Nuc), 2 recombinant Nuc forms, SP_{Usp45} -Nuc and SP_{Exp4} -Nuc (encoded by *usp_{SP-nuc}* and *exp4_{SP-nuc}* fusions, respectively) are produced under NICE control. **b** Western blot analysis. NZ9000 (pSec:Nuc) and NZ9000 (pLB141) strains were grown exponentially to $OD_{600} = 0.4$ and 1 ng/ml nisin was added for 1 h. Proteins from cells (C) and supernatant (S) fractions were analyzed by SDS-PAGE and Western blotting using anti-Nuc antibodies. Precursor forms (SP_{Usp45} -Nuc and SP_{Exp4} -Nuc, both referred to as pre.), primary mature and secreted forms (resulting from SP_{Usp45} or SP_{Exp4} processing, both referred to as NucB) and secondary mature forms (resulting from HtrA processing, referred to as NucA) are shown.



A New Expression/Secretion System. P_{Zn} *zitR* expression system and SP_{Exp4} signal peptide have been cloned together, leading to a new expression/secretion vector. pLB145 was used to produce and secrete a recombinant Nuc form (SP_{Exp4} -Nuc; fig. 6a). Induction is achieved in IL1403 (pLB145) strain grown in GM17 medium at 30°C by addition of 1 mM EDTA at $OD_{600} = 0.1$. Coomassie staining shows that recombinant Nuc secretion into the medium is efficient as amounts of Nuc forms exceed those of Usp45: Nuc yield in the medium reaches 10 mg/l/ OD_{600} after 4 h of induction (fig. 6b). This is close to yields previously reported for SP_{Usp45} -Nuc fusion using

either the NICE system (20 mg/l/ OD_{600} after induction by 1 ng/ml nisin) [Bermudez-Humaran et al., 2003a] or the P170 system (5 mg/l/ OD_{600} at pH = 5.2) [Madsen et al., 2000] under similar conditions (in GM17 medium), even though optimized fermentation processes and possibly chemically defined medium could greatly improve yields [Mierau et al., 2005b; Ravn et al., 2000]. The P_{Zn} *zitR* SP_{Exp4} expression/secretion system is thus a new effective tool to produce and secrete heterologous proteins in *L. lactis*, either in a wild-type (fig. 6b) or an *htrA* mutant strain (data not shown).

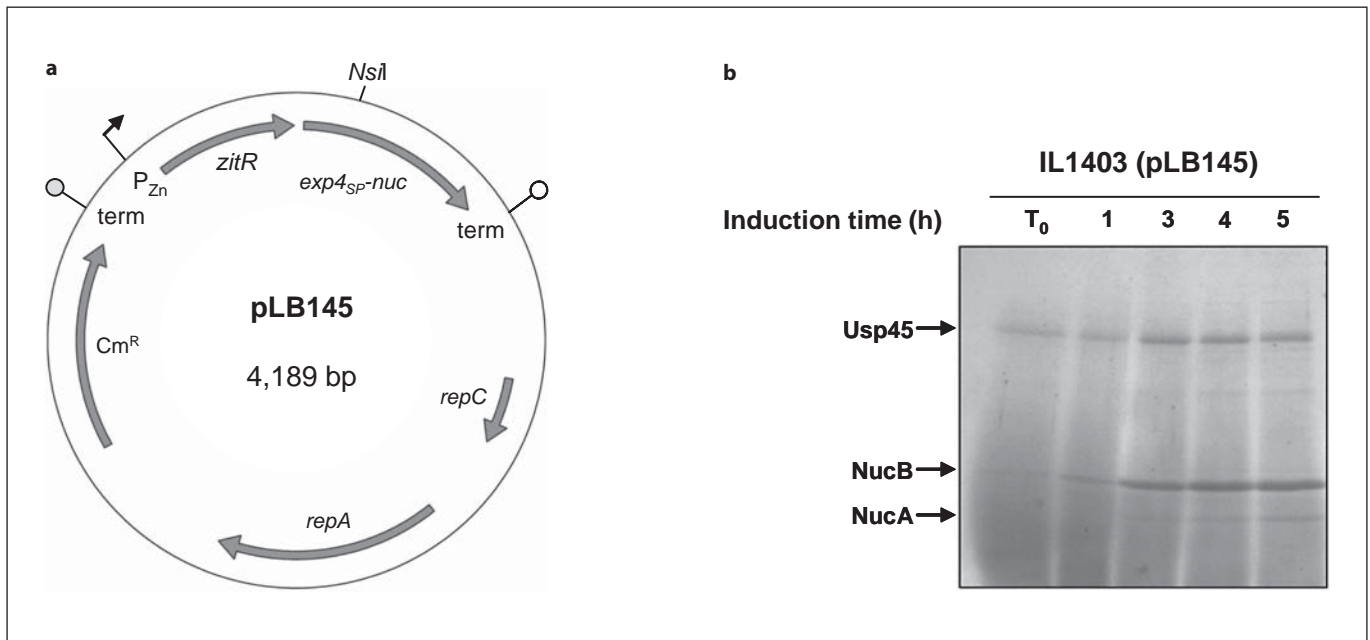


Fig. 6. Staphylococcal nuclease production using the P_{Zn} *zitR* SP_{Exp4} expression/secretion system. **a** pLB145 expression/secretion vector. pLB145 is a pLB141 derivative where the P_{nisA} promoter region has been replaced by P_{Zn} *zitR* to control the expression of *exp4_{SP-nuc}* (encoding SP_{Exp4} -Nuc). The unique *NsiI* restriction site allows cloning of any open reading frame as a translational fusion to SP_{Exp4} . **b** Coomassie brilliant blue staining analysis. IL1403 (pLB145) strain was grown on GM17 medium at

30°C until $OD_{600} = 0.1$ (T_0). At T_0 , EDTA was added to a final concentration of 1 mM. Supernatant samples were taken at T_0 and after 1, 3, 4 or 5 h of induction, precipitated and loaded on SDS-PAGE. The background smear results from medium peptones precipitation. Major proteins in the medium are indicated by arrows: lactococcal Usp45 protein and the staphylococcal Nuc forms, NucB and NucA (primary and secondary mature forms of SP_{Exp4} -Nuc, respectively).

Conclusions

L. lactis is an attractive host to produce and secrete heterologous proteins. To further promote its biotechnological use, we are developing a toolbox comprising an expression/secretion system and a set of improved host strains. A tightly regulated expression system (P_{Zn} *zitR*) was combined in 1 vector with a consensus secretion signal (SP_{Exp4}) and used in a mutant strain (*htrA*) that assures protein stability at the cell surface. *B. subtilis* SecDF accessory protein might also be used in the case of problematic recombinant proteins. In the future, several tools reported to enhance protein secretion and production should be combined into a single strain. Our system is compatible with large-scale production processes and downstream purification steps should be simple and thus low cost.

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