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Research Letter – Food Microbiology

Heterologous expression and purification of the phage lysin-like bacteriocin LysL from *Lactococcus lactis* LAC460

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Abstract

The wild-type *Lactococcus lactis* strain LAC460 produces two bacteriocin-like phage lysins, LysL and LysP. This study aimed to produce and secrete LysL in various heterologous hosts and an *in vitro* cell-free expression system for further functional studies. Initially, the *lysL* gene from *L. lactis* LAC460 was cloned into *Lactococcus cremoris* NZ9000 and *L. lactis* N8 strains, with and without the *usp45* signal sequence (SS_{usp45}), under a nisin-inducible promoter. Active LysL was primarily produced intracellularly in recombinant *L. lactis* N8, with some secretion into the supernatant. Recombinant *L. cremoris* NZ9000 lysed upon nisin induction, indicating successful *lysL* expression. However, fusion with Usp45 signal peptide (SP_{Usp45}–LysL) weakened LysL activity, likely due to incomplete signal peptide cleavage during secretion. Active LysL was also produced *in vitro*, and analysed in SDS-PAGE, giving a 42-kDa band. However, the yield of LysL protein was still low when produced from recombinant lactococci or by *in vitro* expression system. Therefore, His-tagged LysL was produced in *Escherichia coli* BL21(DE3). Western blot confirmed the intracellular production of about 44-kDa His-tagged LysL in *E. coli*. His-tagged active LysL was then purified by Ni-NTA affinity chromatography yielding sufficient 4.34 mg of protein to be used in future functional studies.

Keywords: *Lactococcus lactis*; phage lysin; heterologous expression; Usp45 signal peptide; protein purification; inhibitory tests

Introduction

Lactococcus lactis is a lactic acid bacterium broadly applied as starter culture for fermentation of dairy products. Thus far, more than 40 different bacteriocins, ribosomally synthesized antimicrobial proteins, have been characterized from *Lactococcus* spp. (Takala et al. 2023). Bacteriocins are generally divided into three classes, although alternative classifications have also been proposed (Soltani et al. 2021). Nearly all lactococcal bacteriocins belong to either class I or II bacteriocins, heat-stable peptides. Class I bacteriocins mostly act by cell membrane disruption and inhibition of peptidoglycan synthesis (Simons et al. 2020). Class II bacteriocins cause cell death through different ways, mainly by disrupting membrane permeability (Lozo et al. 2017). To date, the only class III bacteriocins, heat-labile large antimicrobial proteins, identified from lactococci are prophage-encoded lytic enzymes LysL and LysP from *L. lactis* LAC460 (Takala et al. 2023).

Lytic enzymes so far characterized from *Lactococci* are autolysins and phage-related lysins, which either contribute to autolysis or behave like bacteriocins (Visweswaran et al. 2017, Takala et al. 2023). To date, there are four main *N*-acetylglucosaminidases (AcmA, AcmB, AcmC, and AcmD) and one peptidase (YjgB) identified in *Lactococcus* spp. genomes. *N*-acetylglucosaminidases are mostly involved in cell separation and are involved in cell autolysis (Visweswaran et al. 2013). YjgB is an endopeptidase, which degrades the cell wall of lactococci (Redko et al. 2007). More recently, Usp45, the major extracellular protein of *Lactococcus* spp. was also reported to act as a peptidoglycan hydrolase that mediates cell separation (Hernandez-Valdes et al. 2020). As Usp45 is the most secreted protein in *Lactococcus* spp., its signal peptide (SP_{Usp45}) has been used for producing many different heterologous proteins in lactococci (Wan et al. 2015, Bıyıklı et al. 2023).

Prophages and prophage-like elements are present in all lactococcal genomes (Aucouturier et al. 2018). For instance, Kelleher et al. (2018) studied 30 strains of *Lactococcus* spp., and all of them carried 2–10 intact, questionable, or incomplete prophage regions. All intact and some incomplete lactococcal prophages carry genes encoding lytic enzymes called virion associated lysins (VALs) and endolysins (Fernandes and São-José 2018, Abdelrahman et al. 2021). VALs are phage lysins with both enzymatic and structural functions. They help perforate the peptidoglycan layer of target bacteria, allowing the viral genome to enter the target cells. Endolysins, in contrast, act in the end of lytic cycle to degrade the peptidoglycan of the host bacteria (Abdelrahman et al. 2021). Endolysins are usually coexpressed with holins, small proteins facilitating the access of endolysins to peptidoglycan by forming pores in the cell membrane (Saier and Reddy 2015). This process is followed by the lysis of the host cell, ultimately leading to the release of phages. As demonstrated previously (de Ruyter et al. 1997), heterologous expression of lactococcal phage lysins in cheese starter strains accelerates cheese ripening by releasing intracellular enzymes from the lysed starter bacteria. This could be accomplished by producing lysins in a lysin-sensitive cheese starter strain, followed by the lysis of the host, or by secreting the lysins from a lysin-resistant adjunct starter strain.

In our previous work, we found a prophage-encoded bacteriocin-like lysin, LysL, from the culture supernatant of *L. lactis* strain LAC460 (Takala et al. 2023). LysL is a 385-aa lysozyme + peptidase M23 from a defective prophage. Among

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34 *Lactococcus* strains tested, 11 were sensitive to LysL, while it had no lytic effect on the producer strain. However, there is no experimental evidence about the precise mode of action of LysL or its secretion mechanism. Therefore, in order to study the function of LysL, expression and purification of the enzyme are needed.

In this study, the *lysL* gene was cloned and the LysL protein was produced in different heterologous hosts, as well as expressed *in vitro*. In addition, LysL protein was purified, and the secretion of LysL was investigated.

Materials and methods

Plasmids, strains, and culture conditions

Plasmids and bacterial strains used in this study are listed in Table 1. *Escherichia coli* strains were grown in lysogeny broth (LB) medium (1% tryptone, 0.5% yeast extract, and 1% NaCl) with shaking at 37°C. *Lactococcus lactis* and *L. cremoris* strains were incubated in M17 medium (Oxoid Ltd. Basingstoke, UK) supplemented with 0.5% (w/v) glucose (M17G) at 30°C. Solid media were made by adding 1.5% agar to the liquid media. For selecting *Lactococcus* transformants, erythromycin was used at a concentration of 10 µg/ml (M17GE). For *E. coli* transformants, 250 µg/ml erythromycin or 150 µg/ml ampicillin was used.

DNA techniques

Gene amplifications were carried out by standard PCR (Eppendorf, Hamburg, Germany) and using Phusion High-Fidelity DNA polymerase (Thermo Scientific, Waltham, MA, USA), according to the instructions of the manufacturers. PCR primers used are listed in Supplementary Table S1. DNA purification of PCR products and from agarose gel were carried out using the GeneJET PCR Purification Kit and GeneJET Gel Extraction Kit (Thermo Scientific), respectively. Plasmid DNA was isolated from the recombinant cultures with the GeneJET Plasmid Miniprep Kit (Thermo Scientific). Transformation of the strains was done by electroporation with Gene Pulser (Bio-Rad Laboratories, Hercules, CA, USA). PCR products were sequenced by an outsourced DNA sequencing service in the Institute of Biotechnology (University of Helsinki, Finland).

Construction of expression vectors

The *lysL* gene and SS_{usp45} were amplified by PCR using *L. lactis* LAC460 culture as template. SS_{usp45} was fused upstream of *lysL* (SS_{usp45}–*lysL*) using gel-extracted amplicons of the previous PCRs as templates in overlap extension PCR (OE-PCR). The vector composed of the replication genes *repAC*, the erythromycin resistance gene *ermC*, and the nisin-inducible promoter P_{nisZ} was amplified by PCR from plasmid pWUST25 (Wan et al. 2016). The vector fragment was cut with *NcoI*/*ApaI* restriction enzymes, and the *lysL* and SS_{usp45}–*lysL* fragments with the compatible enzymes *BspHI*/*ApaI* (Thermo Scientific) at 37°C for 30 min, as recommended by the supplier. Digested and purified DNA fragments were ligated using T4 DNA ligase (Thermo Scientific) overnight at room temperature, as instructed by the manufacturer. Figure 1 illustrates the construction of *lysL* expression plasmid (pLEB823) and SS_{usp45}–*lysL* expression plasmid (pLEB824). The ligation mixtures were transferred by electroporation into *E. coli* TG1, essentially according to Zabarovsky and Winberg (1990). Colonies were screened for the right constructs by PCR with P_{nisZ} F and RSF (*repAC* screening forward) primers (Supplementary Table S1). Plasmids were isolated from the right clones, followed by sequencing to verify correct constructs. The *lysL* expression plasmids pLEB823 and pLEB824, as well as the control plasmid pVS2 were then electroporated into *L. cremoris* NZ9000 and *L. lactis* N8, essentially as described by Holo and Nes (1989). *Lactococcus cremoris* NZ9000 strains carrying SS_{usp45}–*lysL* expression plasmid, *lysL* expression plasmid, or pVS2 were named as LAC474, LAC475, and LAC476, respectively. *Lactococcus lactis* N8 strains carrying SS_{usp45}–*lysL* expression plasmid, *lysL* expression plasmid, or pVS2 were named as LAC477, LAC478, and LAC479, respectively.

Activity assay

Lactococcus cremoris LAC277 was chosen as the indicator for LysL activity tests because it is LysL sensitive, and nisin and erythromycin resistant without nisin production. Inhibitory activity of the recombinant *L. lactis* N8 strains carrying the *lysL* expression plasmids against *L. cremoris* LAC277 was determined using spot-on-lawn method as described by Wan et al. (2015). Briefly, five times sterile water-diluted overnight culture of *L. cremoris* LAC277 in M17GE was spread and left to dry onto an M17GE agar plate. Cell-free supernatant (CFS) of the overnight sample cultures were obtained by centrifugation at 5000 × *g* for 10 min followed by filtering through 0.22 µm sterilized filter. 10 ml of CFSs were concentrated using 30 kDa centrifugal filters (Merck, Darmstadt, Germany) according to manufacturer's instructions. After centrifuging (5000 × *g*, 10 min), the upper phase was washed twice with 5 ml of PBS, and finally centrifuged to a final volume of 500 µl.

The centrifuged cell pellets of the overnight sample cultures were used for obtaining the cell lysates. After centrifugation of the cultures, the pellets were resuspended in the same volume of PBS (pH 7.2). 1 ml of each cell suspension was added to a 2-ml tube containing 0.1 mm glass beads (Omni International, Kennesaw, GA, USA) and placed in Omni bead mill homogenizer (Bead Ruptor Elite, Omni International). The cell breakage was performed at the speed of 6 m/s for 40 s at 0°C. The tubes' contents were then centrifuged at 4000 × *g* for 10 min, and the cell lysates were obtained by passing each supernatant through 0.22 µm sterilized filter. 10 µl of the concentrated CFS or cell lysate samples were then spotted on the indicator plates and incubated at 30°C overnight.

In the case of *L. cremoris* NZ9000, the recombinant colonies were cultivated in M17GE and incubated at 30°C until the OD_{600} value reached to 0.5 (about 5 h). The induction was then applied with nisin at the concentration of 0.5 IU/ml and the incubation was continued for another few hours.

In vitro expression

Cell-free protein biosynthesis of LysL was done using the rapid translational system (RTS) 100 *E. coli* HY Kit (Biotechrabbit GmbH, Berlin, Germany). Using the forward primer *lysL* F OE T7 carrying overlap extension to T7 promoter, with the reverse primer *lysL* R OE RTS (Supplementary Table S1), *lysL* containing homologous regions of the RTS forward adaptor sequence at the 5′ ends, and RTS reverse adaptor sequence at the 3′ ends was amplified. The purified PCR product was then used as a template for the second PCR with the primers RTS T7 F and RTS R (Supplementary Table S1), which bear RTS forward and RTS reverse adaptor sequences, respectively. Thereby, T7 promoter and RTS forward adaptor sequences were added upstream of *lysL* gene, and the RTS reverse adaptor sequence was fused downstream of that, forming the RTS linear expression template. Finally, 0.5 µg of the purified expression template was used for *in vitro* protein synthesis reaction in a volume of 50 µl. The reaction was incubated for 6 h at 30°C according to the manufacturer's instructions. One neutral reaction was also run in parallel without template, serving as the control.

Table 1. Plasmids and bacterial strains used in this study.

Name	Description	Reference
pET-22b(+)	*E. coli* expression vector harboring pBR322 *ori*, *ampR*, PT7	GenScript Biotech (Leiden, Netherlands)
pLEB823	*lysL* expression plasmid for *Lactococcus*; *repAC*, *ermC*, P_{nisZ}, *lysL*	This study
pLEB824	SS_{usp45}–*lysL* expression plasmid for *Lactococcus*; *repAC*, *ermC*, P_{nisZ}, SS_{usp45}–*lysL*	This study
pLEB844	6x*His*lysL in pET-22b(+) under PT7 control	This study
pVS2	Control plasmid; *repAC*, *ermC*	von Wright et al. (1987)
pWUST25	*Lactococcus* expression plasmid; source of *repAC*, *ermC*, P_{nisZ}	Wan et al. (2016)
E. coli BL21(DE3)	Protein production strain; IPTG inducible gene expression	Studier and Moffatt (1986)
E. coli ECO859	BL21(DE3) carrying *lysL* expression plasmid pLEB844	This study
E. coli TG1	Cloning host	Sambrook et al. (2001)
L. lactis LAC460	Wild-type LysL producer; source of *lysL* gene	Takala et al. (2023)
L. cremoris LAC277	Indicator strain; resistant to nisin and erythromycin, sensitive to LysL	Takala et al. (2004)
L. cremoris NZ9000	Nisin inducible gene expression host; sensitive to LysL	Kuipers et al. (1998)
L. cremoris LAC474	NZ9000 carrying pLEB824; SS_{usp45}–*lysL* expression, Erm^R	This study
L. cremoris LAC475	NZ9000 carrying pLEB823; *lysL* expression, Erm^R	This study
L. cremoris LAC476	NZ9000 carrying pVS2; control strain, Erm^R	This study
L. lactis N8	Constitutive gene expression host with nisin promoter; nisin producer, resistant to LysL	Valio Ltd, Helsinki, Finland; (Wan et al. 2021)
L. lactis LAC477	N8 carrying pLEB824; SS_{usp45}–*lysL* expression, Erm^R	This study
L. lactis LAC478	N8 carrying pLEB823; *lysL* expression, Erm^R	This study
L. lactis LAC479	N8 carrying pVS2; control strain, Erm^R	This study

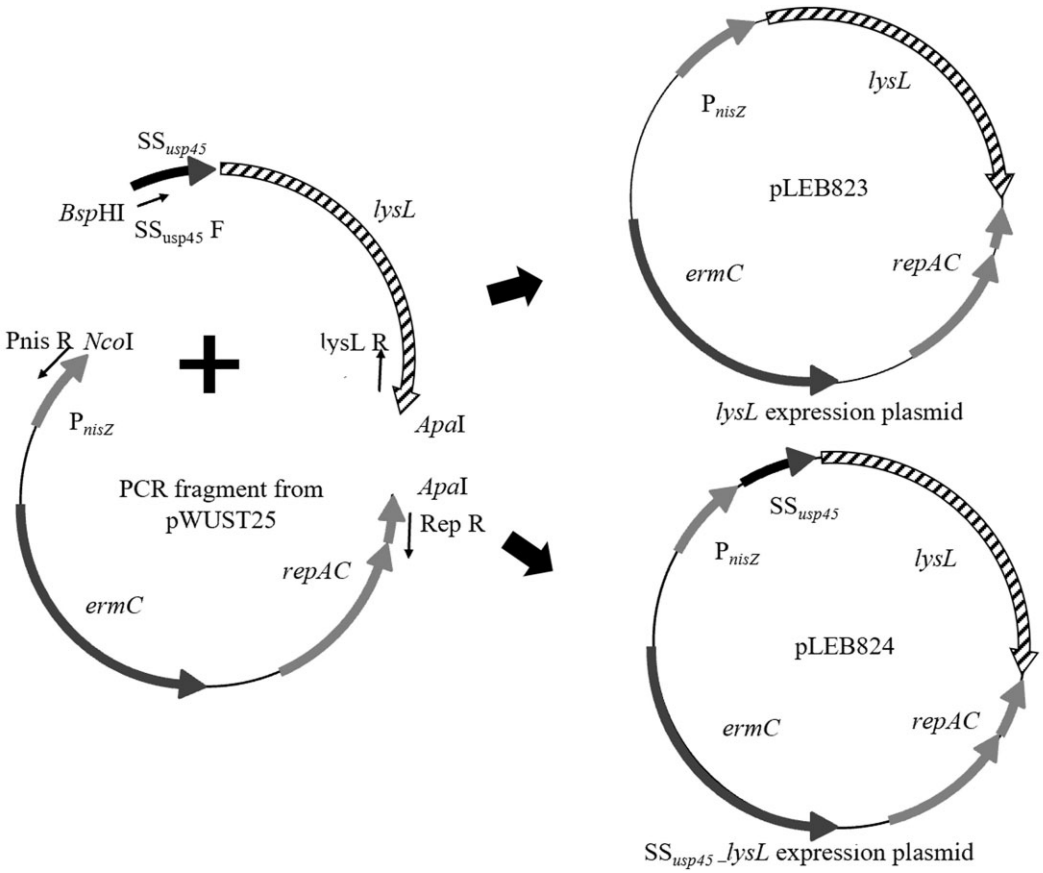

Figure 1. Schematic overview of the construction procedure of the lactococcal *lysL* expression plasmid pLEB823, and the SS_{usp45}–*lysL* expression plasmid pLEB824. *ermC*, erythromycin resistance gene; *repAC*, plasmid replication genes; and P_{nisZ}, nisin-inducible promoter.

SDS-PAGE

SDS-PAGE was performed according to the method essentially described by Laemmli with modifications (Laemmli. 1970). The *in vitro* translation reactions were mixed with SDS-PAGE loading buffer (Bio-Rad Laboratories) and heated at 95°C for 5 min. 10 µl of the samples were then loaded to a gradient SDS-PAGE gel (4%–20%; Bio-Rad Laboratories) and electrophoresed at 4°C under 200 V for 30 min. The gel was then fixed in a fixing solution (100 mM ammonium acetate, 10% acetic acid, and 50% methanol) for 30 min with gentle agitating. It was then stained

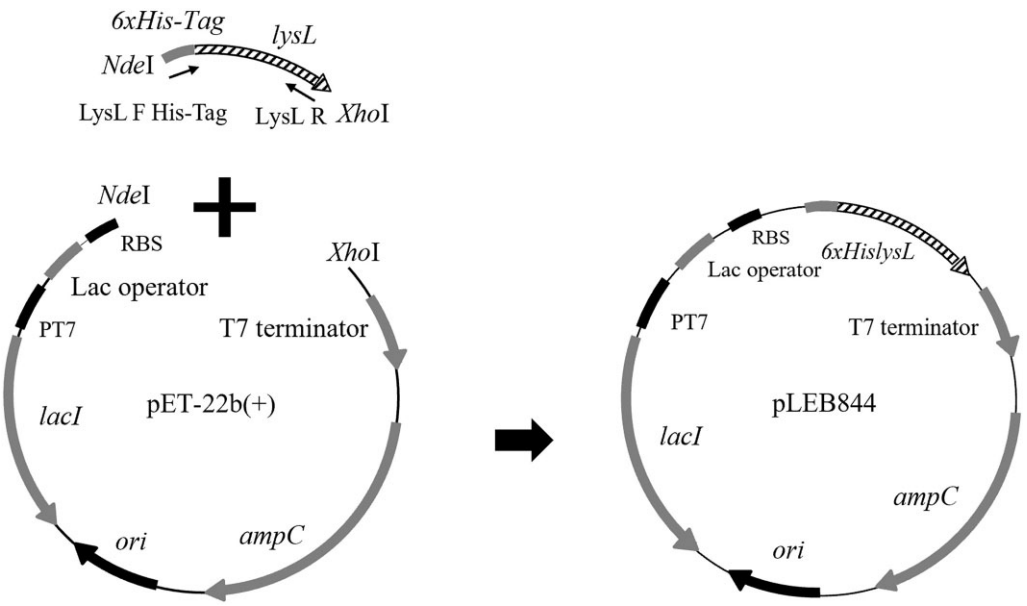

Figure 2. Construction of the *E. coli lysL* expression plasmid pLEB844. The *lysL* gene was amplified, digested, and ligated into the pET-22b(+) backbone via *NdeI*/*XhoI* restriction sites. The vector contains T7 promoter and ampicillin resistance gene, *ampC*.

with Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories) for 1 h, followed by destaining for 2 h in a 10% acetic acid solution.

Cloning of *lysL* gene into the pET-22b(+) vector

Heterologous expression of *lysL* in *E. coli* BL21(DE3) was done using pET-22b(+) as the expression vector. The gene *lysL* was amplified using the primers LysL F His-Tag Xa *NdeI* and LysL R *XhoI* (Supplementary Table S1), which amplify *lysL* with 6 × His codons plus the Factor Xa protease cleavage site for removal of His-Tag in the N-terminus of LysL. The vector and insert were then cut using *NdeI*/*XhoI* restriction enzymes at 37°C for 30 min and ligated, resulting in the plasmid pLEB844 (pET-22b(+)-*lysL*) (Fig. 2). Then, the ligation mixture was electroporated into *E. coli* BL21(DE3) similarly as described above for *E. coli* TG1. Colony PCR screening using T7 F and LysL R *XhoI* primers (Supplementary Table S1), followed by sequencing of the insert, confirmed correct construct. The recombinant strain was stored as *E. coli* ECO859.

Western blot

200 ml LB Amp^{150} was inoculated with an overnight incubated seed of *E. coli* ECO859, then incubated at 37°C with shaking (200 rpm). The induction of protein expression was performed by adding 1 mM isopropylthio-β-galactoside (IPTG) after OD_{600} reached about 0.5. The incubation was then continued for protein production for another 20 h under the same conditions. The cells were then collected by centrifugation (5000 × *g*, 10 min). CFS was prepared by filtering the supernatant through 0.22 µm sterilized filter. The cell pellet was dissolved in 50 ml of PBS (pH 7.2) and lysed four times with an Emulsiflex C3 high-pressure homogenizer (Avestin Inc., Ottawa, Canada). Then, cell debris was separated by centrifugation (30 000 × *g*, 4°C, 20 min).

Western blotting was done according to Towbin et al. (1979) and Burnette (1981) by the Protein Service core facility of the Tampere University. SDS-PAGE electrophoresis was performed for 30 µl of the CFS and the pellet lysate, excluding the staining and destaining steps. Briefly, the samples were separately mixed with Tris–glycine SDS-PAGE loading buffer (Bio-Rad Laboratories) with the ratio of 5:1 and heated at 95°C for 5 min. 5 µl of the samples were then loaded into a gradient SDS-PAGE gel (AnykD; Bio-Rad Laboratories) and electrophoresed at room temperature under 200 V for 30 min. The protein bands were electrotransferred from SDS gel to a cellulose nitrate membrane under 120 V at 4°C for 40 min. The membrane was then incubated in blocking solution (10% (w/v) bovine serum albumin) for 1 h with mild agitation. Next, the membrane was probed with 1:10 000 diluted in 5% Milk-TBST primary mouse anti-His.H8 (Thermo Scientific) at 4°C overnight with mild agitation. It was then incubated with horse antimouse IgG(H + L) Peroxidase (Vector Laboratories, Oxfordshire, UK) at a 1:20 000 dilution in TBST buffer (0.1% Tween 20, 25 mM Tris, 150 mM NaCl, pH 7.5) at room temperature for 1 h. After the last three steps, the membrane underwent three washes, each lasting 5 min, with TBST buffer. Protein bands were lastly detected using Western-Bright ECL HRP substrate (Advansta, San Jose, CA, USA) with the ChemiDoc MP Imaging System (Bio-Rad Laboratories) according to the producer's instructions.

Protein purification

LysL originating from 900 ml of IPTG-induced culture of *E. coli* ECO859 was purified with affinity chromatography using 6 × His-Tag as the purification tag by outsourced protein purification service (Protein Service core facility of the Tampere University). Prior to the chromatography step, 10 mM imidazole was separately added to the prepared CFS and pellet lysate samples (previous section) to prevent unspecific binding. The samples were then bound to HisPur™ Ni-NTA agarose (Thermo Scientific) in a batch mode for 1 h at 4°C. The bound protein was washed with 16 column volumes of wash buffer (PBS, 250 mM NaCl, 50 mM imidazole, pH 7.2) before stepwise elution with Elution Buffer (PBS, 250 mM NaCl, pH 7.2), which had an increasing concentration of imidazole (100, 150, 200, and 250 mM). The concentration (mg/ml) of the protein was obtained using the equation C = c(molar) × MW, in which, c(molar) = Abs280 nm/extinction coefficient.

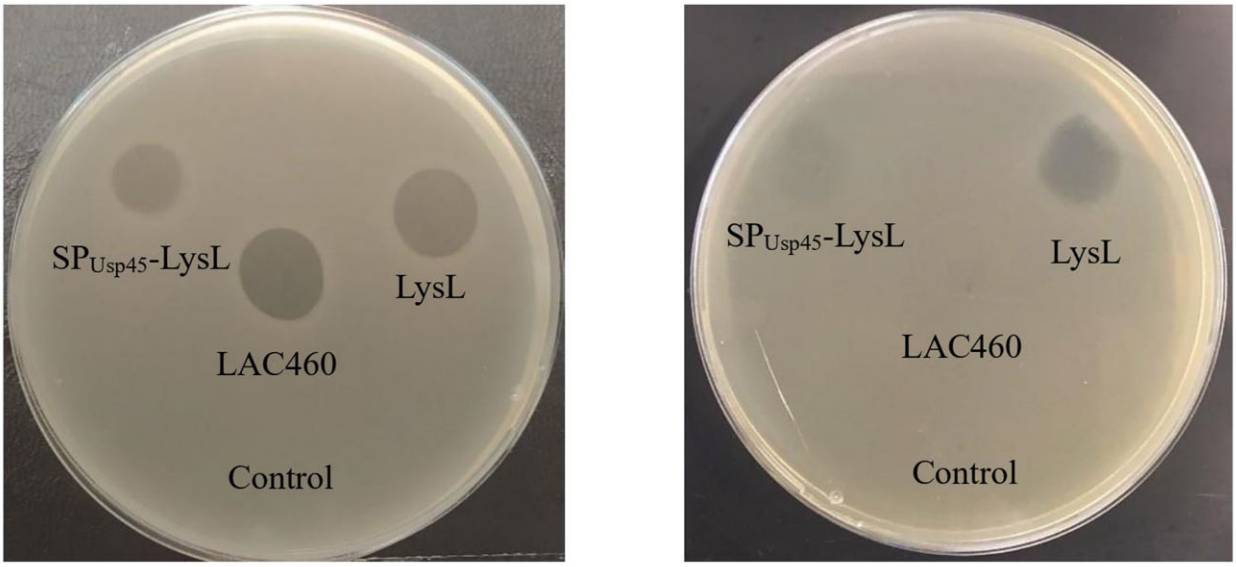

Figure 3. Inhibition of *L. cremoris* LAC277 by LysL from recombinant *L. lactis* N8 and wild-type *L. lactis* LAC460 concentrated CFSs (A) and cell lysates (B). 10 µl of the lysates/20-fold concentrated CFSs were spotted onto *L. cremoris* LAC277 indicator lawn on M17GE and incubated overnight at 30°C. LysL, *L. lactis* N8 transformed with the *lysL* expression plasmid; SP_{Usp45}–LysL, *L. lactis* N8 transformed with the SS_{usp45}–*lysL* expression plasmid; LAC460, *L. lactis* LAC460; and control, *L. lactis* N8 transformed with pVS2. A clearer and larger halo was created by the CFS of LysL producing *L. lactis* N8 than that of SP_{Usp45}–LysL producer. The cell lysate containing LysL caused a clear inhibition zone whereas the SP_{Usp45}–LysL lysate created a very diffuse halo. In case of LAC460, only CFS gave inhibition halo. The figure was digitally processed to enhance their quality.

Results

Heterologous expression of *lysL* in *Lactococcus* spp

As LysL originates from *L. lactis*, and the nisin-inducible heterologous gene expression method for lactococci is known to be strong, we aimed to overproduce LysL in *Lactococcus* strains. The *lysL* gene was cloned in *L. cremoris* NZ9000 and *L. lactis* N8, which carry nisin regulatory genes needed for gene expression with the nisin promoter. Also, in order to secrete LysL to the supernatant of the recombinant cultures, SS_{usp45} was fused with the *lysL* gene (SS_{usp45}–*lysL*), and parallel to *lysL*, was cloned into the bacteria under the control of a nisin-inducible promoter.

As shown in Fig. 3(A), both concentrated CFSs from the recombinant *L. lactis* N8 cultures gave halos on the indicator plates, demonstrating that the expression of *lysL*, both with and without SS_{usp45}, was successful, and active LysL was secreted to supernatant. The halo formed by SP_{Usp45}–LysL was weaker than that of native LysL.

Next, the cells were lysed to investigate the activity of LysL and SP_{Usp45}–LysL inside the cell. As seen in Fig. 3(B), the cell lysate containing LysL gave a clear halo on the indicator plate, while SP_{Usp45}–LysL resulted in much weaker inhibition. Hence, either most of the SP_{Usp45}–LysL protein was secreted, or the activity of the SP_{Usp45}–LysL fusion is lower than native LysL. The strong halo from concentrated LAC460 CFS but not from its lysate shows that in the wild-type strain LAC460, LysL is completely secreted. However, when expressed in heterologous *L. lactis* N8, most of the produced LysL is found inside the cells, as the clear halo observed in Fig. 3(A) results from 20-fold concentrated CFS, representing about 200 µl supernatant, while the halo in Fig. 3(B) represents cells from about 10 µl culture.

The attempt to produce LysL in the LysL-sensitive strain *L. cremoris* NZ9000 caused lysis of the producer after nisin induction. Figure 4 shows the lysis extent of NZ9000 producing LysL and SP_{Usp45}–LysL after induction with nisin. Compared with the control NZ9000(pVS2), the culture of LysL producing NZ9000 turned completely clear within 80 min, whereas SP_{Usp45}–LysL producing strain lysed only partially. Further incubation of the latter culture also resulted in complete lysis (result not shown). The slower lysis could be explained by incomplete cleavage of SP_{Usp45} during secretion, decreasing the activity of LysL. The result is comparable with that of *L. lactis* N8, where native LysL presented higher activity in CFS than when fused with SP_{Usp45}. It can be concluded that in heterologous host, LysL is partly secreted without additional signal peptide, and that it is active both inside and outside the cell. Therefore, LysL could be produced in sensitive strain for the host autolysis, but not for purification purposes, as the secreted enzyme lyses the host too fast.

In vitro expression and identification of LysL

Even though LysL produced from heterologous *L. lactis* N8 was highly active and functional, the protein production level was still low for studying the mode of action of the enzyme. Therefore, we tested whether the cell-free *in vitro* gene expression system would give higher yield of LysL. The *lysL* template with T7 promoter and the adaptor sequences for the *in vitro* system was produced by OE-PCR, and the reaction was performed in 50 µl volume. Supplementary Figure S2 shows the comparison of the activity of LysL produced from different sources. The *in vitro* system could produce more LysL than the heterologous *L. lactis* N8. Since the expression in the cell-free translational system was stronger, the lysin produced *in vitro* was chosen for visualization on SDS-PAGE gel. The reaction solution of *in vitro* expression with *lysL* template, as well as the reaction solution without template as a control were electrophoresed in a gradient gel. Since the RTS 100 *E. coli* HY Kit is based on *E. coli* lysate, other intracellular *E. coli* proteins are also

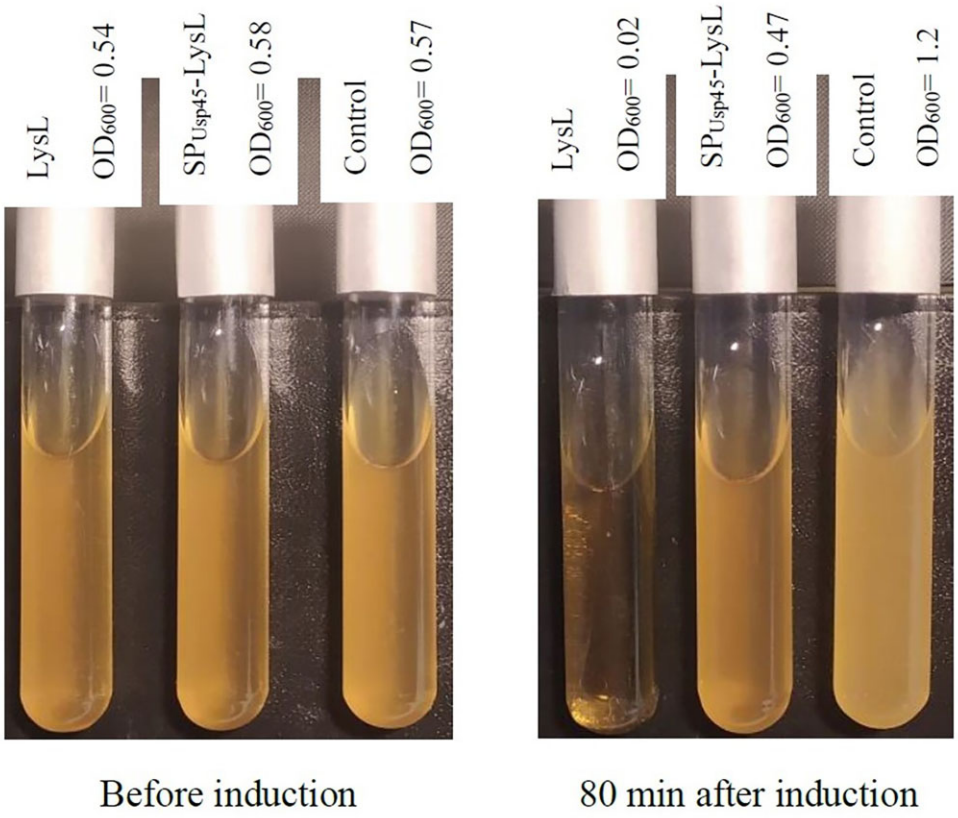

Figure 4. Autolysis of the LysL-producing recombinant *L. cremoris* NZ9000 cultures before and 80 min after induction by nisin. LysL, *L. cremoris* NZ9000 producing native LysL; SP_{Usp45}–LysL, *L. cremoris* NZ9000 producing SP_{Usp45}–LysL; and control, *L. cremoris* NZ9000 carrying plasmid vector pVS2. After 5 h (OD_{600} about 0.5) incubation at 30°C, the cultures were induced by nisin (0.5 IU/ml) followed by 80 min incubation. Native LysL-producing culture turned completely transparent, whereas the SP_{Usp45}–LysL-producing culture remained partly clear. The control strain culture without any LysL production became turbid. The figure was digitally processed to enhance their quality.

present in addition to the translated ones. As shown in Fig. 5(A), LysL (translated from *lysL*) presents the only additional band in the gel, when compared with the control. As marked in lane 1 the band is in the expected size of about 42 kDa. The result identifies the LysL protein, and further indicates that the antimicrobial activity of the cell-free translational reaction solution comes from LysL.

Production of LysL in *E. coli* and purification of the recombinant protein

Although *in vitro* production of LysL was higher than that by the *Lactococcus* recombinant strains, the quantity versus costs was not adequate for further investigation of the mode of action. In order to obtain LysL in adequate quantity for further studies, LysL expression was carried out in *E. coli* and later purified by affinity chromatography using a His-Tag. The sequence encoding 6 × His was added to the 5′-end of *lysL*, ligated with the vector pET-22b(+), and cloned into *E. coli* BL21(DE3). Prior to purification, Western blot was carried out to confirm the intracellular location of LysL, as well as to confirm the presence of the N-terminal 6 × His-Tag in the produced LysL. The cell lysate obtained from overnight culture of *E. coli* ECO859 was used for anti-His antibody detection. The strong distinct band at about 44 kDa represents LysL carrying the 6 × His-Tag recognized by anti-His antibody (Fig. 5A).

LysL was purified using 6 × His-Tag and HisPur™ Ni-NTA chromatography. The optimum concentration of imidazole for elution of the bound protein in the last step was found to be 100 and 150 mM. From the 900 ml of the initial culture of *E. coli* ECO859, the total purified protein was 4.34 mg at a concentration of 0.7 mg/ml. The activity test of the purified enzyme showed that it was active (Supplementary Figure S3).

Discussion

In this study, we expressed *L. lactis* prophage lysin gene *lysL* in different heterologous hosts and in *in vitro* cell-free gene expression system. Production of active LysL enzyme was successful both in *Lactococcus* spp. and cell-free *in vitro* system. However, the protein yields obtained were insufficient for conducting further functional studies. Consequently, LysL was then produced and purified from recombinant *E. coli* cells.

Phage lysins have previously been expressed in many heterologous bacteria, including *E. coli* and *Lactococcus* spp. for various purposes. For example, phage lysins can be produced in cheese starter strains to lyse the starter cells for speeding up the cheese maturation step (de Ruyter et al. 1997). Still, the interest in phage lysins has more commonly been focused on producing lysins from phages of pathogenic or spoilage bacteria, and using the lysins as specific antimicrobials. For instance, prophage-encoded endolysin LysP108 from *Staphylococcus aureus* XN108, and several phage structural genes from *Klebsiella* bacteriophage ΦK64-1 have been expressed in *E. coli*, showing specific antimicrobial activity against the target bacteria (Lu et al. 2021 and Pan et al. 2017). As examples of using *Lactococcus* spp. as hosts for heterologous

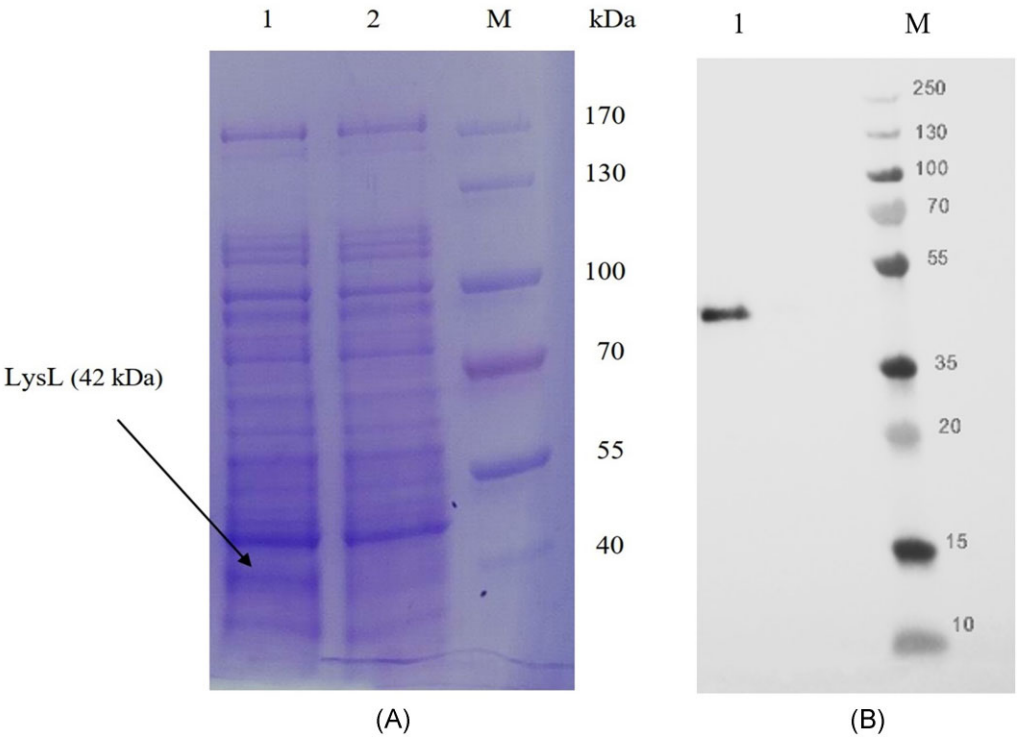

Figure 5. (A) Coomassie blue-stained SDS-PAGE visualizing proteins produced in the cell-free translational system using an RTS 100 *E. coli* HY Kit. Lane 1, reaction solution using *lysL* as a template; Lane 2, reaction solution without template as a control; and M, PageRuler Protein Ladder (Thermo Scientific). The figure was digitally processed to enhance their quality. (B) Western blot analysis of the cell lysate of the culture of *E. coli* BL21(DE3) carrying pET-22b(+)-*lysL*. The His-Tag antibody used recognized LysL containing a 6 × His-Tag in the N-terminus (lane 1), M, PageRuler Broad Range Protein Ladder (Thermo Scientific).

lysins, *L. cremoris* NZ9000 and *L. lactis* INIA 415 have been used to produce endolysins Endo88 from *S. aureus* bacteriophage 88, and CTP1 L from *Clostridium tyrobutyricum*, respectively (Garde et al. 2020, Chandran et al. 2022). Similar to our study here, these *Lactococcus* studies used nisin promoter to drive the lysin gene expression; constitutive expression in the nisin producing strain INIA 415, and nisin inducible production in the strain NZ9000. The latter strain is undoubtedly the most commonly used *Lactococcus* for nisin controlled gene expression, also known as the NICE system (Mierau and Kleerebezem 2005). Nisin induction is generally regarded as strong and tightly regulated, and therefore overproducing active LysL in another *Lactococcus* strain felt a reasonable and safe strategy, in terms of e.g. avoiding protein misfolding. Tight regulation of the gene expression was indeed demonstrated, as the apparent suicide gene *lysL* could easily be cloned under nisin promoter in LysL sensitive NZ9000. However, the induced production of LysL enzyme was highly detrimental for the host, and hence large-scale production of the protein would be impossible. Nevertheless, successful production and secretion of LysL in different *Lactococcus* strains demonstrated its potential usefulness in cheese making. Production of LysL in a LysL-sensitive starter strain or a LysL-resistant adjunct starter would be expected to cause the release of starter cells' cytosolic enzymes to the cheese matrix for accelerated cheese ripening.

To overproduce LysL in heterologous *Lactococcus*, we aimed to try both intracellular and extracellular production. We hypothesized that addition of the signal peptide of Usp45 to the N-terminus of LysL would improve its secretion, which could also be required for the activity of LysL. SP_{Usp45} has been widely used for secreting homologous and heterologous proteins in lactococci and other bacteria. For instance, β-1,3–1,4-glucanase from *Bacillus* sp. SJ-10 (Tak et al. 2019) and glucansucrase from *Leuconostoc mesenteroides* (Skory and Côté 2015) have been secreted from the recombinant *Lactococcus* strains by SP_{Usp45}. Replacement of the native signal peptide with SP_{Usp45} has also been reported to enhance secretion of recombinant proteins. For example, after deletion of the signal peptide's inherent sequence in *cbh2*, *bgl1*, and *egl3*, also using SP_{Usp45} instead, the secretion in *L. lactis* was enhanced (Liu et al. 2016). Nevertheless, in some cases, manipulating the signal peptide of the recombinant proteins has impaired secretion. For example, Borrero et al. (2011) reported that, when produced by *E. hirae* DCH5, antimicrobial activity of the recombinant hiracin JM79, in which the inherent signal peptide was replaced by SP_{Usp45}, was decreased. Therefore, although SP_{Usp45} has been proven to facilitate extracellular secretion of recombinant proteins in many studies, there is no guarantee that it is better signal peptide than the native one in all cases. Our study also provided an example for SP_{Usp45} not improving the yield of active enzyme in supernatant, since the fusion of SS_{usp45} with *lysL* in recombinant lactococci caused lower inhibitory activity compared with the corresponding native LysL producers. As shown in Fig. 3(B), the lysate of the nisin producing *L. lactis* host N8 producing LysL created a strong halo on the indicator plate, while the halo from SP_{Usp45}–LysL was significantly fainter. This illustrates that SP_{Usp45} attached to LysL disturbs the activity of LysL, as all LysL in the lysate is expected to be fused with SP_{Usp45}. Hence, it is possible that SP_{Usp45} improved the secretion of LysL but decreased its activity due to incomplete cleavage of the leader. The weak halo from SP_{Usp45}–LysL in Fig. 3(A) probably reflects the portion of LysL in the CFS, which has been secreted through the Sec-dependent pathway, and from which the SP_{Usp45} had been cleaved.

Interestingly, in the wild-type producer strain LAC460, LysL was completely excreted to the environment, as no active LysL was found in the cell lysate (Fig. 3B). The protein seems not to contain a typical signal peptide for secretion, and hence, the mechanism of the secretion is unknown. It is still clear that LysL finds its way out of the LAC460 cells, whereas in the heterologous *L. lactis* host N8, LysL mostly remains inside the cell, suggesting that some factors needed for efficient secretion are missing in the strain N8. As the production of LysL in *L. lactis* N8 turned out to be somewhat ineffective,therefore, we then aimed to produce LysL in a cell-free *in vitro* system.

Cell-free protein synthesis may yield a higher production rate of recombinant proteins than cellular expression systems. The system is also useful for production of toxic proteins, or those that are difficult to express (Jin and Hong 2018), For instance, cell-based production of colicin, a bacteriocin from *E. coli*, requires coproduction of immunity proteins, if produced in *E. coli*, and therefore the overexpression of colicin is more convenient *in vitro* (Jin et al. 2018). In our study, the *in vitro* production of LysL provided sufficiently protein for antimicrobial tests and SDS-PAGE. However, the total protein yield fell short of expectation, and particularly the quantity versus costs was not reasonable for producing large amounts of LysL by *in vitro* system. Therefore, the decision was made to overproduce LysL in *E. coli* as the host. After all, the *E. coli* approach proved to be both straightforward and successful, resulting in significant quantities of active LysL protein suitable for subsequent functional studies with minimal challenges such as protein misfolding or the formation of inclusion bodies. *Escherichia coli* has frequently been used as the host for overexpression of lysins (Wang et al. 2020, Chu et al. 2022). For instance, Dreher-Lesnick et al. (2015) demonstrated the use of recombinant *Lactobacillus* phage lysin LysA2 for purity assays of probiotics, and Chu et al. (2022) highlighted the antimicrobial potential of Abp013 against multidrug-resistant *Acinetobacter baumannii*, with both studies relying on purified lysin.

There are several possible explanations about the secretion mechanism of LysL in LAC460. To date, many protein secretion pathways have been discovered in Gram-positive bacteria, for example the classical Sec and Tat pathways, and at least seven nonclassical secretion mechanisms (Dai et al. 2022). Nonclassically secreted proteins are proteins without a predictable signal sequence or known secretory pathways, but which are still found in the extracellular space. An apparent secretion mechanism of LysL in its native host LAC460 could be through a holin pore, as prophage holins have been reported to mediate nonlytic secretion of proteins, particularly phage endolysins (Mukherjee et al. 2002, Palmer et al. 2021, Brüser and Mehner-Breitfeld 2022). The type of holin determines whether it contributes to transferring proteins into the cell exterior through secretion, leakage, or membrane lysis (Desvaux 2012).

In any case, dozens of nonclassically secreted proteins have been reported from *Lactococcus*, *Listeria*, *Streptococcus*, and other Gram-positive bacteria (Wang et al. 2016). Perhaps LysL is another nonclassical secretory protein, and its mechanism of secretion remains to be investigated.

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Supplementary data

Supplementary data is available at *FEMSLE Journal* online.

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