Engineering *Lactococcus lactis* as a multi-stress tolerant biosynthetic chassis by deleting the prophage-related fragment

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**Abstract**

**Background:** In bioengineering, growth of microorganisms is limited because of environmental and industrial stresses during fermentation. This study aimed to construct a nisin-producing chassis *Lactococcus lactis* strain with genome-streamlined, low metabolic burden, and multi-stress tolerance characteristics.

**Results:** The Cre-loxP recombination system was applied to reduce the genome and obtain the target chassis strain. A prophage-related fragment (PRF; 19,739 bp) in the *L. lactis* N8 genome was deleted, and the mutant strain *L. lactis* N8-1 was chosen for multi-stress tolerance studies. Nisin immunity of *L. lactis* N8-1 was increased to 6500 IU/mL, which was 44.44% higher than that of the wild-type *L. lactis* N8 (4500 IU/mL). The survival rates of *L. lactis* N8-1 treated with lysozyme for 2 h and lactic acid for 1 h were 1000- and 10,000-fold higher than that of the wild-type strain, respectively. At 39 °C, the *L. lactis* N8-1 could still maintain its growth, whereas the growth of the wild-type strain dramatically dropped. Scanning electron microscopy showed that the cell wall integrity of *L. lactis* N8-1 was well maintained after lysozyme treatment. Tandem mass tags labeled quantitative proteomics revealed that 33 and 9 proteins were significantly upregulated and downregulated, respectively, in *L. lactis* N8-1. These differential proteins were involved in carbohydrate and energy transport/metabolism, biosynthesis of cell wall and cell surface proteins.

**Conclusions:** PRF deletion was proven to be an efficient strategy to achieve multi-stress tolerance and nisin immunity in *L. lactis*, thereby providing a new perspective for industrially obtaining engineered strains with multi-stress tolerance and expanding the application of lactic acid bacteria in biotechnology and synthetic biology. Besides, the importance of PRF, which can confer vital phenotypes to bacteria, was established.

**Keywords:** *Lactococcus lactis*, Prophage-related fragment, Genome editing, Multi-stress tolerance, Nisin immunity, Nisin yield, TMT quantitative proteomics

**Background**

The microbial cell factory, which mainly involves microorganisms to produce organic acids, chemicals, and antimicrobial peptides, is used for various purposes in industrial biotechnology [1]. Lactic acid bacteria (LAB) are commonly used as microbial cell factories [2] and *Lactococcus lactis* (*L. lactis*) is particularly employed to produce the food additive (E234) nisin. However, microorganisms often encounter environmental stress, including oxidative, acid, and heat stresses [3]. Moreover, nisin producers have to tolerate the stress caused by the antibacterial nisin. To date, only limited studies have reported on the construction of microbial chassis *L. lactis* strains to enhance multi-stress tolerance, reduce
metabolic burden, and boost desirable product fermentation. Therefore, there is an urgent need to engineer LAB in order to adapt to extreme environmental pressures and accomplish normal growth and high production of valuable compounds [4].

In recent years, researchers have adapted strains with stress tolerance through different strategies, e.g., random mutagenesis, global transcription machinery engineering (gTME), global regulator overexpression and genome editing. Random mutagenesis has been extensively used to improve the acid tolerance of microbial cells. The global regulator Sigma D factor (RpoD) of *Escherichia coli* (*E. coli*) had been tailored by mutagenesis to achieve enhanced acid resistance, and the best mutant exhibited much higher growth rate than the control (0.22 h⁻¹ vs 0.15 h⁻¹) at pH 3.17 [5]. The ethanol tolerance of *Saccharomyces cerevisiae* (*S. cerevisiae*) had been increased after gTME by reprogramming gene transcription [6]. The survival rate of *E. coli* had been noted to increase by 10- to 100-fold at pH 2.5 by overexpressing the global regulator H-NS [7]. Li et al. overexpressed sHSP20 in *E. coli* BL21 cells and increased its survival period at 50 °C by almost 2 h [8], while deletion of *ADY2* improved the growth of *S. cerevisiae* under acetic acid, ethanol, and hydrogen peroxide stresses [9].

The two major methods to improve stress tolerance of LAB are heterologous expression [10] and endogenous overexpression [11]. In addition, genome shuffling has also been applied to improve acid tolerance and volumetric productivity of an industrial strain *Lactobacillus rhamnosus* ATCC 11443 [12]. Besides, small RNA like sRNA-s015 [13] has also been reported to be an important factor that affects stress tolerance. Recently, the resistance or survival rates of *L. lactis* have been improved by exposing the bacterial cells to transglutaminase [14], bacteriocin Lcn972 [15], and Tween 80 [16] during growth.

In general, deletion of non-essential genome fragments, such as prophages and transposons, could confer some advantages on bacterial strains [17], including enhanced growth, increased biomass, and higher level of proteins synthesis [18]. Besides, prophages can usually synthesize proteins like prophage lysin that affect the host cell wall/membrane [19], and cell wall/membrane is essential for maintaining cellular integrity and resisting environmental stress [20]. For instance, the structure of the cell wall/membrane of nisin-producing *L. lactis* can also affect its tolerance to nisin [21]. Therefore, deletion of prophage-related fragments (PRFs) might affect multi-stress tolerance, nisin immunity, and nisin production of LAB. However, studies on the multi-stress tolerance of LAB are limited. For example, in a previous study, a putatively prophage-deleted derivative of *L. lactis* UC509 was constructed and the type and integrity of the prophage were investigated [22]. Likewise, *L. lactis* IL1403 derivatives were constructed and all the PRFs were deleted; however, the functions of these prophages in the strain were not explored [23]. Furthermore, a chassis derivative of *L. lactis* NZ9000 was generated, which exhibited superior growth phenotype and higher heterologous protein synthesis, but did not present multi-stress tolerance [4]. It must be noted that the above-mentioned studies did not include nisin-producing strains. The level of nisin production by a nisin producer could be regulated by the producer’s immunity to nisin [24]. Thus, to ensure growth and efficient production, *L. lactis* N8 must have the ability to resist nisin inhibition. In general, the nisin immunity of a producer is determined by different mechanisms, namely, structure of cell wall/membrane [21], local pH at the outer surface of the cytoplasmic membrane [25], nisin-digesting enzyme [26], and nisin immunity genes [27].

Bacterial tolerance is a complex regulatory mechanism. Before the advancement of proteomics technology, there was no systematic research method to understand bacterial tolerance at the protein level. Two-dimensional gel electrophoresis (2-DE) is a traditional method to identify “upregulated” and “downregulated” proteins [28]. However, 2-DE has limitations such as low resolution and bias against membrane proteins. In recent years, mass spectrometry (MS) technology has been widely used for the analysis of complex protein mixtures, and MS-based proteins quantitative analysis has replaced 2-DE proteomics. When compared with transcriptomics gene expression studies, proteomics can directly measure the level of gene products in a specific state and can further characterize protein activity, interaction, and subcellular distribution [29]. Proteomics has been successfully applied in various fields, such as determination of protein composition of organelles, elucidation of protein–protein interactions, and large-scale mapping of protein phosphorylation in response to stimuli [30]. Tandem mass tags (TMT)-labeled MS for protein synthesis analysis has been widely used to study the tolerance mechanism of microorganisms [29].

In the present study, TMT-labeled proteomics technology was applied to trace the changes in the protein synthesis of mutant strains. Functional analysis of differentially synthetized proteins was performed, and the protein response mechanism was elucidated based on enrichment results. The mechanism of multi-stress tolerance of the strain was also elaborated at the proteome level, and the results obtained can provide a basis for subsequent research in related fields. A PRF was deleted from *L. lactis* N8 genome using the Cre-loxP recombination system [31], and the mutant *L. lactis* N8-1
outperformed the wild-type strain in several physiological traits assessed, including better stress tolerance and higher nisin yield in acidic fermentation. TMT quantitative proteomics showed that the upregulated proteins were mainly enriched in sugar metabolism and biosynthesis of cell wall and cell surface proteins. The findings of this study provide new strategies for developing industrial strains with increased multi-stress tolerance and a novel perspective for the acquisition of highly robust strains.

Results

**Design and construction of L. lactis N8-1**

To construct the mutant strains, PHAST (PHAge Search Tool) was used for PRF prediction (http://phast.wishartlab.com/) [32]. The genome of L. lactis N8 contains seven PRFs (about 0.231 Mb), constituting 8.98% of the L. lactis N8 genome (about 2.57 Mb) (Fig. 1a). By using the Cre-loxP recombination system, one PRF was deleted. The location of the deleted PRF in L. lactis N8 is shown in Fig. 1b, and the genetic components of the deleted PRF are presented in Fig. 1c. The deleted PRF was 19.7 kb and contained 22 open reading frames. Among these genes, 14 have been characterized and one encodes phage integrase. A detailed description of the genes is provided in Additional file 1: Table S1. The deletion result was verified by PCR with confirmation primers and genome resequencing (Fig. 1d, e).

**Mutant strain showed increased nisin immunity**

Analysis of the nisin immunity of the L. lactis N8-1 showed that there was significant difference between the mutant and wild-type strains in nisin immunity (Fig. 2a, b). L. lactis N8-1 presented 44.44% higher nisin resistance than L. lactis N8 (Fig. 2c). The growth curves of the wild-type and mutant strains were obtained under different concentrations of nisin, and revealed that there were no significant differences in growth between the mutant and wild-type strains at 4000 IU/mL (Fig. 2d), 5000 IU/mL (Fig. 2e), and 6000 IU/mL (Fig. 2f) nisin concentrations. These results indicated that the wild-type and mutant strains could grow in GM17 medium with high concentration of nisin [6500 IU/mL (Fig. 2g), 7000 IU/mL (Fig. 2h), and 7500 IU/mL (Fig. 2i)]; however, the wild-type strain started to grow was 15–20 h later than the mutant strain, suggesting that L. lactis N8-1 took less time to overcome the side effects of nisin and start to grow. These findings indicated that L. lactis N8-1 has better nisin immunity than L. lactis N8. Moreover, although nisin producers were not killed by high concentrations of nisin to a certain extent, their growth was still restrained.

**Mutant strain exhibited increased resistance to lysozyme**

The lysozyme tolerance of L. lactis N8 and L. lactis N8-1 was evaluated by drop plate experiments. The survival rates of L. lactis N8 and L. lactis N8-1 after lysozyme treatment were determined to clarify the difference between both the strains. The L. lactis N8-1 exhibited higher survival rates after treated with lysozyme at various time points (Fig. 3a). After treatment for 60 min, L. lactis N8-1 exhibited 75.3-fold higher survival rate, when compared with L. lactis N8. Moreover, after treatment for 120 min, the survival rate of L. lactis N8-1 was markedly higher (1000-fold) than that of the control strain (Fig. 3b). These results demonstrated that loss of PRF conferred L. lactis N8-1 lysozyme tolerance.

**Mutant strain presented increased resistance to lactic acid**

To determine lactic acid tolerance, the growth curves of L. lactis N8 and L. lactis N8-1 under different concentrations of lactic acid were acquired (Fig. 3). There was no significant difference between the OD600 of L. lactis N8 and L. lactis N8-1 when the concentration of lactic acid was 0.24% (v/v) (Fig. 3e). In contrast, when the concentration of lactic acid was increased to 0.32% (v/v), the OD600 of the mutant strain was 58% higher than that of the wild-type strain after 18 h of incubation (Fig. 3f). The mutant strain continued to grow, whereas the wild-type strain could hardly grow when the lactic acid concentration was increased to 0.40% (v/v) (Fig. 3g). Drop plate experiment showed that L. lactis N8-1 exhibited higher survival rates at various time points after being treated with lactic acid [1.5% (v/v)], and the survival rate of L. lactis N8-1 was significantly higher than that of L. lactis N8 (10,000-fold) after 60 min of treatment (Fig. 3c, d).

**Mutant strains reached higher cell density at high temperature**

To determine the tolerance of the mutant strain to high temperature, the growth curves of L. lactis N8 and L. lactis N8-1 under different temperatures were acquired. There was no significant difference between the OD600 of L. lactis N8 and L. lactis N8-1 when the temperatures were 30 °C (Fig. 3h) and 37 °C (Fig. 3i). However, when the temperature was raised to 39 °C, significant difference in the OD600 was observed between L. lactis N8 and L. lactis N8-1 (Fig. 3j). After 9 h, the growth of L. lactis N8 drastically decreased, while L. lactis N8-1 could still grow. The growth curves obtained at 39 °C revealed abnormal L. lactis N8 growth, indicating that high temperature had disrupted its normal growth; in contrast, the growth of L. lactis N8-1 was still normal.
Mutant strains showed higher utilization of several carbon sources

Extensive fermentation phenotype analyses of *L. lactis* N8-1 and *L. lactis* N8 were conducted using the phenotype microarrays to further understand the physiological differences between the wild-type and mutant strains. Additional file 1: Table S2 summarizes the comparisons of substrates consumed by *L. lactis* N8-1 and *L. lactis* N8. The results showed that the mutant strain could efficiently metabolize...
some substrates, including α-D-glucose, D-mannose, sucrose, and N-acetyl-D-glucosamine, which are associated with carbon source metabolism and cell wall biosynthesis. The mutant *L. lactis* N8-1 exhibited distinct properties with respect to metabolism of four carbon sources, when compared with the wild-type strain.

**Fig. 2** The nisin immunity and the growth profiles of *L. lactis* N8 and *L. lactis* N8-1 at GM17 medium supplemented with different concentrations of nisin. **a** 96-well plate gradient dilution cultures; **b** the result of the OD600 value of bacterial culture achieved by microplate reader; **c** nisin immunity of *L. lactis* N8 and *L. lactis* N8-1. **d** Growth curves at 4000 IU/mL nisin; **e** growth curves at 5000 IU/mL nisin; **f** growth curves at 6000 IU/mL nisin; **g** growth curves at 6500 IU/mL nisin; **h** growth curves at 7000 IU/mL nisin; **i** growth curves at 7500 IU/mL nisin.
Fig. 3  The survival rates and growth profiles of \textit{L. lactis} N8 and \textit{L. lactis} N8-1 under different conditions. \textbf{a} Gradient dilution drop plate experiment treated with lysozyme (10 mg/mL). \textbf{b} Survival rate curves treated with lysozyme (10 mg/mL). \textbf{c} Gradient dilution drop plate experiment treated with lactic acid (1.5% (v/v)). \textbf{d} Survival rate curves treated with lactic acid (1.5% (v/v)). \textbf{e} Growth curves at 30°C; \textbf{f} growth curves at 37°C; \textbf{g} growth curves at 39°C; \textbf{h} Growth curves under 0.24% (v/v) lactic acid; \textbf{i} growth curves under 0.32% (v/v) lactic acid; \textbf{j} growth curves under 0.40% (v/v) lactic acid.
Scanning electron microscopy of cells after lysozyme treatment

To determine the causes of multi-stress tolerance of the mutant \textit{L. lactis} N8-1, the morphology and cell integrity of \textit{L. lactis} N8 and \textit{L. lactis} N8-1 after lysozyme treatment were examined by scanning electron microscopy (SEM). As shown in Fig. 4, following lysozyme treatment (17.5 mg/mL) for 60 min, the cell wall of \textit{L. lactis} N8-1 maintained better integrity, whereas that of \textit{L. lactis} N8 presented obvious holes. After 120 min of lysozyme treatment, the cell wall of \textit{L. lactis} N8-1 was damaged, while that of \textit{L. lactis} N8 was almost completely degraded. These results (from a micro-perspective) revealed that the cell wall of the mutant \textit{L. lactis} N8-1 was more resistant to lysozyme, and confirmed that \textit{L. lactis} N8-1 exhibited better robustness.

\textbf{Fig. 4} Scanning electron microscopy (SEM) of \textit{L. lactis} N8 and \textit{L. lactis} N8-1 treated with lysozyme (17.5 mg/mL)
Mutant strains achieved higher nisin yield under acidification conditions

To obtain strains with superior nisin immunity and nisin production, we constructed engineered strains to obtain a high nisin yield chassis. However, after overexpressing the nisin immunity genes, \textit{nisFEG}, \textit{nis-IFEG}, and \textit{nisRFEG}, in \textit{L. lactis} N8-1, we were unable to obtain mutant strains with higher nisin immunity characteristics (Additional file 1: Table S3). As strains used in industrial production are known to endure acid stress, we used lactic acid to adjust the pH of the medium for constant-pH fermentation. As shown in Fig. 5a, there was no obvious difference in nisin production between \textit{L. lactis} N8 and \textit{L. lactis} N8-1 when the pH of the medium was 6.0. However, when the medium pH was adjusted to 5.5, the nisin yield of \textit{L. lactis} N8-1 was 36.29% higher than that of \textit{L. lactis} N8 at 12 h (Fig. 5b). When the pH of the medium was adjusted to 5.0, \textit{L. lactis} N8 had limited growth with no increased nisin production, while the growth of \textit{L. lactis} N8-1 was significantly restrained, but presented considerable nisin production (Fig. 5c). Subsequently, we overexpressed \textit{nisZ} in \textit{L. lactis} N8-1 and \textit{L. lactis} N8 and found that the nisin yield of \textit{L. lactis} N8-1 was 22.57% and 18.79% higher than those of the wild-type strain at 10 and 12 h, respectively (Fig. 5d). These results indicated that \textit{L. lactis} N8-1 is more advantageous than \textit{L. lactis} N8 as a microbial cell factory with higher nisin yield and nisin immunity.

TMT quantitative proteomics analysis and transcription verification

The mutant strain \textit{L. lactis} N8-1 with improved nisin immunity and multi-stress tolerance (lysozyme, lactic acid, and high-temperature tolerance) was constructed. Based on the results of TMT quantitative proteomics, a total of 2164 proteins were detected. Different databases were used to annotate proteins, while KEGG database was used to classify the proteins (Fig. 6a, b). We found that 33 proteins were significantly upregulated and nine proteins were significantly downregulated (> 1.5-fold change with \(P<0.05\)) in \textit{L. lactis} N8-1, when compared with those in \textit{L. lactis} N8 (Fig. 6c). The upregulated proteins and downregulated proteins (> 1.2-fold change with a \(P<0.05\)) are listed in Additional file 1: Tables S4, S5, respectively. KEGG functional enrichment analysis (\textit{L. lactis} N8-1 vs \textit{L. lactis} N8) showed that the upregulated proteins were mainly related to sugar metabolism and amino acid biosynthesis/metabolism (Additional file 1: Fig. S1a). In contrast, the downregulated proteins were mainly related to ribosome biosynthesis (Additional file 1: Fig. S1b). The gene ontology (GO) function annotations for all proteins (and upregulated and downregulated proteins) are shown in Additional file 1: Fig.
S2. Protein subcellular localization analysis showed that the upregulated proteins were mainly sub-located in the cytoplasm and extracellular (43 proteins), whereas the downregulated proteins were mainly sub-located in the cytoplasm (34 proteins) (Additional file 1: Fig. S3). Subsequently, several genes with higher and lower expression levels were examined by real-time quantitative PCR (RT-qPCR) to verify the proteomics data. When compared with \textit{L. lactis} N8, the expression levels of genes \textit{rpsN}, \textit{rplR}, \textit{csc2B}, \textit{csc2C}, \textit{pi339}, \textit{pp423}, \textit{butA}, \textit{butB}, \textit{arcA}, \textit{arcB}, \textit{galM}, \textit{galK} and \textit{lac2} were altered in \textit{L. lactis} N8-1 (about 0.37-, 0.32-, 2.55-, 3.17-, 3.00-, 3.79-, 3.52-, 2.20-, 3.51-, 2.37-, 4.49-, 5.60-, and 2.55-fold, respectively) (Additional file 1: Fig. S4), which were in agreement with the results of proteomics assay.

**Discussion**

In laboratory or industrial fermentation process, microbial strains are generally believed to endure many harsh conditions, including oxidation, heating and cooling, acid, high osmolarity/dehydration, and starvation [33]. Therefore, understanding of the stress response behavior of \textit{L. lactis} is of crucial significance for expanding its application in industrial fermentation. In recent years, an increasing number of studies have confirmed that \textit{L. lactis} can survive by activating specific protection mechanisms in response to environmental pressure [34]. To tolerate heat shock, \textit{L. lactis} regulates the folding and maturation of new or denatured proteins through the synthesis of heat shock proteins (HSPs). Genetic analyses have confirmed the presence of the conserved HSPs in \textit{L. lactis}, including \textit{DnaK}, \textit{DnaJ}, \textit{GroEL}, \textit{GroES}, and \textit{GrpE} [33]. Under low temperature pressure, \textit{E. coli} can prevent the formation of secondary structures in RNA molecules and stimulate translation efficiency by regulating the cold shock proteins (CSPs), \textit{CspA} [35]. Researchers have identified conserved CSPs (\textit{CspA}, \textit{CspB}, \textit{CspC}, \textit{CspD}, and \textit{CspE}) in \textit{L. lactis} and proved the important role of these CSPs under cold shock conditions [36]. Under low pH condition, LAB mainly employ two mechanisms to
respond to stimuli. The primary mechanism for the control of intracellular pH is F$_{0}$F$_{1}$ ATPase that translocates protons to the environment at the expense of ATP. Both the expression level and activity of this protein complex have been noted to increase at low pH [37]. The second mechanism in response to low pH is the arginine deiminase pathway that allows _L. lactis_ to neutralize its environment through NH$_{3}$ production [38]. To overcome oxidative stress, some _L. lactis_ strains have been reported to accumulate glutathione and possibly utilize glutathione to remove O$_{2}^{-}$ [39, 40]. Conversely, the expression of _recA_ has been noted to be induced in aerated cultures and a _recA_ mutant _L. lactis_ has been found to be highly sensitive to aeration, indicating that _recA_ gene clearly plays a role in oxidative stress [41]. In the past decades, numerous studies have been conducted to improve strain stress tolerance, and heterologous expression and overexpression in LAB have gained much research attention. Sugimoto et al. constructed multi-stress tolerant (3% NaCl, 5% ethanol, and 0.5% lactic acid) _L. lactis_ NZ9000 by heterologous expression of _dnaK_ gene from _E. coli_ [42]. Hagi et al. proved that the heterologous expression of _crtNM_ in _L. lactis_ MG1363 led to the increased H$_{2}$O$_{2}$, low pH, 20% bile acid, and 12 mg/mL lysozyme tolerance [43]. Furthermore, Desmond et al. improved stress tolerance by overexpressing _GroESL_ in _Lactobacillus paracasei_ NFBC 338 [solvent tolerance, the ability to grow in the presence of butanol (0.5% v/v) for 5 h] [11], while Zhu et al. overexpressed ABC transporters in _L. lactis_ NZ9000 and obtained enhanced acid-stress tolerance derivatives [44]. The common strategy that had been applied in these studies is the so-called “plus” method to achieve high synthesis level of the target proteins. In contrast, large non-essential fragments, such as prophages, transposons, and genomic islands, as exogenously inserted chromosomal sequence, are not necessary for the host cells. It has been reported that deletion of large non-essential fragments produced the phenotypes with reduced metabolic burden, faster growth, and increased biomass [4]. Therefore, the “minus” method could be considered as a promising approach.

In the present study, after deleting a PRF, the mutant strain _L. lactis_ N8-1 did not show growth deficiency, when compared with the wild-type strain. Besides, the genome of the mutant strain _L. lactis_ N8-1 was streamlined, which is beneficial for the construction of engineered LAB microbial cell factories. The mutant _L. lactis_ N8-1 exhibited significantly improved nisin immunity, which may provide a good basis for achieving high production of nisin. Proteomics analysis showed that the synthesis of NisI and NisFEG proteins was not increased in the mutant strain. Subsequently, we tried to overexpress the genes _nisFEG, nisIFEG_ and _nisRKFEG_ in _L. lactis_ N8-1; however, no strains with significantly higher nisin immunity were obtained, indicating that beyond a certain range, nisin immunity tends to be under a global and complex regulation.

Besides the substantial increment in nisin immunity, the mutant strains also presented heat, lactic acid, and lysozyme resistances. We speculated that the improved multi-stress tolerance of the mutant strains was owing to the loss of PRF, resulting in remodeling of the cell wall/membrane and changes in carbon source metabolism. Previous studies have shown that the structure of the cell wall/membrane affects the nisin tolerance of LAB [45], and cell wall, as the first barrier of bacteria, is essential for the strain’s stress tolerance [20]. Similarly, in the present study, the synthesis levels of enzymes related to cell wall/membrane biosynthesis and degradation in _L. lactis_ N8-1 were noted to be altered. Thus, combined with the results of lysozyme treatment experiment and SEM, these evidences demonstrated that deletion of PRF resulted in remodeling of the mutant’s cell wall/membrane. Based on the possible pleiotropic changes caused by PRF knock-out, the proteins that led to stress tolerance in the mutant strain could be discussed in the following three groups: (1) carbohydrate and energy transport/metabolism; (2) synthesis of peptidoglycan (PG), cell wall, and cell surface proteins; and (3) others (Table 1). The findings of this study could help us to further understand the possible mechanism of stress tolerance of _L. lactis_, and lay a foundation to explore the deeper and detailed underlying reasons in the future as well as provide significant guidelines for the construction of robust microbial cell factories.

1. Carbohydrate and energy transport/metabolism

Carbohydrate and energy transport/metabolism are the basic life activities of bacteria. Changes in the synthesis of enzymes that catalyze these processes often produce a huge impact on the bacterial phenotype. The proteomics results of the present study indicated that the protein synthesis levels of _GalM_ (aldose 1-epimerase), _GalK_ (galactokinase), and _GalT_ (galactose-1-phosphate uridylyltransferase), which belong to galactose metabolism pathway, were significantly upregulated in _L. lactis_ N8-1. The upregulation of these proteins allowed the metabolic flow to α-D-glucose 1-phosphate, which can be used as an intermediate substrate to participate in glycolysis, pentose and glucurionate interconversions, etc. As the intermediate product of glycolysis, pyruvate participates in sugar metabolism, amino acid metabolism, citrate cycle, and fatty acid metabolism. Previous studies have shown that the _gal_ operon plays a role in the nisin tolerance, and that _L. lactis_ NZ9000△_galAMK_
was twice as sensitive to nisin as its parent strain [46]. However, the contributions of the mechanisms of these transport systems to stress resistance remain to be elucidated. It has been speculated that sugar metabolism intermediates in cell wall biosynthesis or PTS systems might contribute to energy for stress tolerance mechanism [46]. In the present study, the synthesis level of LacZ (β-galactosidase), LacA (galactoside O-acetyltransferase), and LacS (lactose permease) was significantly upregulated in L. lactis N8-1. LacZ catalyzes the hydrolysis of terminal non-reducing β-d-galactose residues in β-d-galactosides, while LacA and LacS are sugar transport proteins. The proteins of this family are major facilitators of membrane transport and may contribute to energy supply in stress tolerance mechanism. Overall, all these mechanisms above could contribute to the ability of the mutant strains to completely utilize carbon source and may provide a basis for the strain’s multi-stress tolerance.

2. Biosynthesis of PG, cell wall and cell surface proteins

Cell wall is essential for stress tolerance in bacteria. Under constantly changing living conditions, enzymes that are involved in PG synthesis and degradation can regulate the dynamic balance of the strain’s cell wall. In L. lactis N8-1, YmjE (glycosyl transferase) and YmjF (UDP-N-acetylglucosamine 2-epimerase), which are essential for the biosynthesis of cell wall surface polysaccharides [47], were significantly upregulated. Cell wall surface polysaccharides have been confirmed to contribute to nisin immunity of LAB [25]. In L. lactis N8-1, synthesis of cell surface proteins, Csc2A, Csc2B, and Csc2C, were significantly upregulated. Siezen et al. speculated that the proteins from cell surface protein complexes may play an important role in carbon source acquisition.

Table 1 Partial upregulated proteins found in proteomic analysis (L. lactis N8-1 vs L. lactis N8)

| Protein name | Description                                           | fc (N8-1/N8) | P-value (N8-1/N8) |
|--------------|-------------------------------------------------------|--------------|-------------------|
| Carbohydrate and energy transport/metabolism          |                                                        |              |                   |
| GalM         | Aldose 1-epimerase                                     | 1.8319       | 0.0328            |
| GalK         | Galactokinase                                          | 1.9828       | 0.0263            |
| GalT         | Galactose-1-phosphate uridylyltransferase              | 1.6774       | 0.0266            |
| LacA         | Galactoside O-acetyltransferase                        | 1.6464       | 0.0307            |
| LacZ         | Beta-galactosidase                                     | 1.7593       | 0.0376            |
| LacS         | Lactose and galactose permease GPH translocator family| 1.5916       | 0.0060            |
| Biosynthesis/degradation of PG, cell wall and cell surface proteins | | | |
| YmjE         | Glycosyl transferase                                   | 1.957        | 0.0187            |
| YmjF         | UDP-N-acetylglucosamine 2-epimerase                    | 2            | 0.0123            |
| YuaE         | Aspartate protease                                     | 1.5713       | 0.0057            |
| ChiA         | Chitinase                                              | 1.6291       | 0.0021            |
| YqcD         | WxL domain-containing protein                          | 2.0262       | 0.0047            |
| Csc2A        | Cell surface protein                                   | 1.5525       | 0.0011            |
| Csc2B        | WxL domain-containing cell surface protein             | 2.1488       | 0.0042            |
| Csc2C        | WxL domain-containing cell surface protein             | 1.8678       | 0.0124            |
| YqbH         | Transcriptional regulator                              | 1.7327       | 0.0358            |
| YbeF         | Collagen binding domain-containing protein              | 2.3745       | 0.0026            |
| Others (phage-related proteins)                       |                                                        |              |                   |
| Pip          | Phage infection protein                                | 1.9826       | 0.0209            |
| NA           | Phage antirepressor                                    | 1.5872       | 0.0176            |
| Pp261        | Uncharacterized protein                                | 1.5545       | 0.0098            |
| P339         | Prophage pI3 protein 39                                | 1.7602       | 0.0087            |
| Pp423        | Phage transcriptional regulator, ArpU family           | 1.9412       | 0.0011            |
| Phi3396      | Phage major capsid protein                             | 1.6566       | 0.0022            |
| NA           | Phage tail protein                                     | 1.9425       | 0.0011            |
| NA           | Phage protein                                          | 1.6534       | 0.0078            |
| P308         | Prophage pI3 protein 34                                | 1.7759       | 0.0092            |
| YaE          | YhgE/Pip (phage infection protein) domain-containing protein | 2.0323 | 0.0212 |
of bacteria [48]. The gene from cscABCD gene cluster encodes WxL-domain-containing cell surface proteins and LPxTG-anchored cell surface proteins. The WxL motif confers prokaryotic cell surface localization function, and this region is the cell wall-binding domain of Gram-positive bacteria and may interact with PG [49]. Besides, the synthesis of YbeF (LPxTG collagen binding domain-containing protein) in L. lactis N8-1 was significantly increased. Pieterse et al. showed that the synthesis of LPxTG-anchored cell surface protein was significantly improved after treatment with lactic acid [50], suggesting that LPxTG-anchored cell surface protein contributes to lactic acid resistance. As these two proteins are anchored on the cell wall/membrane to some extent, we proposed that the cell surface proteins are likely to participate in the stress tolerance of the strain and further protect it. The synthesis and degradation of cell wall is a precise regulation process and maintains certain balance [51]. We speculated that the deletion of PRF could promote remodeling of cell wall, which may eventually make the cell wall become denser or form a protective structure, ultimately providing multi-stress tolerances.

3. Others

In addition to the above-mentioned changes, the synthesis levels of 9 proteins belonging to a complete prophage were significantly upregulated in the mutant strain. Besides, the synthesis of several other bacteriophage infection proteins was also upregulated. A previous study had demonstrated that L. lactis IL1403 acquired stress tolerance (antimicrobial, heme) after deleting all the prophages [23]; however, the study did not clarify the changes in the strain at the transcriptional or translational level; therefore, it is difficult to determine the prophage that conferred stress tolerance to the strain. In the present study, comparison of the PRF deleted from L. lactis N8 with all the PRFs of L. lactis IL1403 revealed no correlation, indicating that deletion of different types of PRF confers diverse characteristics to the strain. However, protein synthesis of a prophage that still remained on the mutant’s genome was increased, suggesting that the absence of a certain type of PRF could affect the protein synthesis of other prophages even in the lysogenic state. It is presumed that recognition and competition mechanisms of the prophages could ultimately affect the phenotype of the strain itself. Thus, it can be concluded that the acquired multi-stress tolerance of the mutant strain L. lactis N8-1 is a very complex trait. Figure 7 summarizes the simplified metabolic pathways. Many proteins possibly involved in stress tolerance were detected and two major mechanisms of acquiring stress tolerance could hypothesized. The first mechanism of acquiring stress tolerance could possibly be through changes in protein synthesis of prophage, with lysophage indirectly contributing to the stress tolerance of the host bacteria; however, detailed reason still needs to be explored. The second and major mechanism could be the changes in the cell wall structure. The cell wall of the L. lactis N8-1 might have probably been altered in two ways: becoming denser (LacS, YmjE, YmjF) and getting thicker (Csc2A, Csc2B, Csc2C).

Conclusions

This study demonstrated that bacterial cells employ diverse mechanisms to defend themselves against multiple stresses. To the best of our knowledge, this is the first report to improve the multi-stress tolerance and nisin immunity of L. lactis by deleting PRF. TMT quantitative proteomics was proven to be an efficient technique to systematically and comprehensively elucidate the possible mechanisms of multi-stress tolerance of PRF knockout strain, which provides a new strategy for industrially obtaining robust L. lactis microbial cell factories with superior tolerance and higher nisin immunity.

Methods

Bacterial strains, plasmids and growth conditions

The strains and plasmids used in this study are listed in Table 2. L. lactis N8 (wild-type nisin Z producer) and its derivatives were cultured at 30 °C without agitation in GM17 medium [M17 broth supplemented with 0.5% (w/v) glucose]. E. coli DH5α cells were grown aerobically at 37 °C in Luria–Bertani (LB) broth (1% tryptone, 0.5% yeast extract, and 1% NaCl) and used as cloning host. Micrococcus luteus NCIB 8166 was cultured aerobically in LB Broth at 37 °C and used as nisin sensitive indicator strain to detect the antibacterial activity of nisin. L. lactis NZ9000 was used as intermediate cloning host for the pNZ8048/pLEB124 plasmids construction, and it was cultured at 30 °C without agitation in GM17 medium. While pNZ8048/pLEB124 was used to construct the expression vector, pNZ5319 was employed to construct the knock-out vector through Cre-loxP gene recombination system. Antibiotics were used when needed: 150 and 5 μg/mL erythromycin for E. coli and L. lactis, respectively; 15 and 5 μg/mL chloramphenicol for E. coli and L. lactis, respectively; 100 μg/mL kanamycin for E. coli; and 20 μg/mL ampicillin for L. lactis.

DNA manipulations and cloning

Restriction enzymes, DNA markers, DNA ligase, DNA polymerases and DNA gel extraction kit were purchased from Takara Bio. Inc. (Dalian, China). Bacterial genome
Fig. 7  Simplified metabolic pathways of L. lactis. The significantly upregulated (> 1.5-fold change with $P < 0.05$) proteins are represented by red frames; upregulated (1.2–1.5-fold change with $P < 0.05$) proteins are represented by blue frames; upregulated (> 1.2-fold change with $P > 0.05$) proteins are represented by purple frames; downregulated (> 1.2-fold change with $P < 0.05$) proteins are represented by green frames.
rapid extraction part A kit was purchased from Spark Jade (China). Polymerase chain reaction (PCR) product purification kit was purchased from Thermo Fisher Scientific (Waltham, USA). The commercial nisin was purchased from Sigma (St. Louis, USA). Chromosomal DNA, plasmid DNA, and total RNA of *L. lactis* N8 or *E. coli* were isolated using QIAprep spin kit (small scale) following the manufacturer’s instructions. PCR was performed on Bio-Rad S1000 Thermal Cycler (Bio-Rad Laboratories, Inc. USA). Primers used in this study were purchased from GENEWIZ (Suzhou, China), and are listed in Table 3. Recombinant plasmids were introduced into *L. lactis* by electroporation using the Bio-Rad Gene Pulser (Bio-Rad Laboratories, Richmond, USA). Plasmids were introduced into *E. coli* through the CaCl₂ method [52].

**Deletion of PRF DNA region**

The vector for the deletion of the PRF was constructed as described in our previous work [53]. Briefly, two fragments (upstream homology arm 1555 bp, downstream homology arm 1414 bp) of the flanking region of PRF were amplified by PCR with a proof-reading polymerase.

| Table 2 Bacterial strains and plasmids utilized in this study |
|---------------------------------|---------------------------------|---------------------------------|
| **Strains or plasmids**         | **Relevant descriptions**       | **Reference**                   |
|---------------------------------|---------------------------------|---------------------------------|
| *E. coli* DH5α                  | Cloning host; F-φ80 lacZΔM15    | [58]                            |
| *E. coli* DH5α-up               | Cm', Em', *E. coli* DH5α derivative containing the whole plasmid pNZ5319-up | This study                      |
| *E. coli* DH5α-up-down          | Cm', Em', *E. coli* DH5α derivative containing the whole plasmid pNZ5319-up-down | This study                      |
| Micrococcus luteus NCB 8166     | Indicator strains for Nisin agar gel diffusion assay | [59]                            |
| L. lactis NZ9000                 | MGI363 pepN:nisRK               | [60]                            |
| L. lactis N8                    | Wild-type (WT) Nisin Z producer | [27]                            |
| L. lactis N8-up-down            | Cm', Em', *L. lactis* N8 derivative containing the whole plasmid pNZ5319-up-down (single crossover) | This study                      |
| L. lactis N8-cat                | Cm', *L. lactis* N8 derivative containing the a lox66-P32-cat-lox71 replacement of PRF (double crossover) | This study                      |
| L. lactis N8-1                  | Mutant. The PRF deletion from *L. lactis* N8 | This study                      |
| L. lactis N8-vector1            | Cm', *L. lactis* N8 derivative containing pNZ8048 | This study                      |
| L. lactis N8-nisZ                | Cm', *L. lactis* N8 derivative containing pNZ8048-nisZ | This study                      |
| L. lactis N8-1-vector1          | Cm', *L. lactis* N8-1 derivative containing pNZ8048 | This study                      |
| L. lactis N8-1-nisZ             | Cm', *L. lactis* N8-1 derivative containing pNZ8048-nisZ | This study                      |
| L. lactis N8-vector2            | Em', *L. lactis* N8 derivative containing pLEB124 | This study                      |
| L. lactis N8-nisFEG              | Em', *L. lactis* N8 derivative containing pLEB672 | This study                      |
| L. lactis N8-nisFEG             | Em', *L. lactis* N8 derivative containing pLEB124-nisFEG | This study                      |
| L. lactis N8-nisRKFEG           | Em', *L. lactis* N8 derivative containing pLEB674 | This study                      |
| L. lactis N8-1-vector2          | Em', *L. lactis* N8-1 derivative containing pLEB124 | This study                      |
| L. lactis N8-1-nisFEG           | Em', *L. lactis* N8-1 derivative containing pLEB672 | This study                      |
| L. lactis N8-1-nisFEG           | Em', *L. lactis* N8-1 derivative containing pLEB124-nisFEG | This study                      |
| L. lactis N8-1-nisRKFEG         | Em', *L. lactis* N8-1 derivative containing pLEB674 | This study                      |
| Plasmids                        |                                  |                                  |
| pNZ5319                         | Cm', Em', used as knock-out vector | [31]                            |
| pNZ5319-up                      | Cm', Em', upstream homology arm amplified from *L. lactis* N8 genome cloned into pNZ5319 | This study                      |
| pNZ5319-up-down                 | Cm', Em', upstream and downstream homology arm amplified from *L. lactis* N8 genome cloned into pNZ5319 | This study                      |
| pNZTS-Cre                       | Em', cre gene cloned at the EcoRI and HindIII sites (cat gene deletion vector) | [53]                            |
| pNZ8048                         | Cm', pNZ8048 derivative containing the promoter P8 | [61]                            |
| pNZ8048-nisZ                    | Cm', pNZ8048 derivative containing the promoter P8/nisZ | [61]                            |
| pLEB124                         | Em', *L. lactis* secretion vector harboring lactococcal promoter P45 | [27]                            |
| pLEB672                         | Em', pLEB124 derivative containing the nisFEG gene | [62]                            |
| pLEB124-nisFEG                  | Em', pLEB124 derivative containing the nisFEG gene | From Per Saris’ lab           |
| pLEB674                         | Em', pLEB124 derivative containing the nisRKFEG gene | [62]                            |
and the L. lactis N8 chromosome was used as template. Then, the fragments were ligated into the XhoI–SwaI and SacI–BglII restriction sites of pNZ5319. The recombinant plasmid pNZ5319-up-down obtained was transformed into E. coli DH5α cells strain by CaCl₂ method [52]. After verifying the accuracy of cloning (Additional file 1: Fig. S5), pNZ5319-up-down was isolated and electroporated into L. lactis N8 competent cells to generate mutant L. lactis N8-1-cat. After the deletion of the PRF, cat gene in the mutant was retrieved by introducing the plasmid pNZTS-Cre into L. lactis N8-1-cat strain. The final mutant was named as L. lactis N8-1 and used for further analysis.

**Genome resequencing**

Isolation of genomic DNA was carried out using SDS method. Total DNA obtained was subjected to quality control by agarose gel electrophoresis and quantified by Qubit (Thermo). The genome of L. lactis N8-1 was sequenced with MPS (massively parallel sequencing) Illumina technology (Illumina, San Diego, USA). The DNA library was constructed: a paired-end library with an insert size of 350 bp. The 350-bp library was sequenced using an Illumina PE150 strategy. Library construction and sequencing were performed at the Beijing Novogene Bioinformatics Technology Co., Ltd. Quality control of paired-end reads were performed using in-house program.

**Data processing**

The original figure data obtained by high-throughput sequencing platform of Illumina PE150 (Illumina) were transformed into raw sequenced reads by CASAVA base calling software (version 1.8.2; Illumina, Inc. USA), and stored in FASTQ format, containing sequencing information and the corresponding sequencing quality information of the reads. The sequenced data were filtered, and the sequence of adapter and low-quality data were removed, resulting in the clean data used for subsequent analysis.

**Reads mapping**

The reads comparison is the basis of the resequencing analysis. The variation information of the sample and the reference is obtained by aligning the sample reads with the designated reference sequence (L. lactis N8 genome). Mapping the reads to the reference sequence using BWA software (version 0.7.17; http://bio-bwa.sourceforge.net), counting the coverage of the reference sequence to the reads and make explanations of the alignment results using the SAMTOOLS software (version 1.11; http://www.htslib.org).

**SV (structural variation) analysis**

SV refers to the insertion, deletion, inversion and translocation of the large segments in the genome level. The insertion, deletion, inversion, intra-chromosomal translocation, and inter-chromosomal translocation between

| Primers | Sequence (5′-3′) |
|---------|-----------------|
| For the construction of deletion vector (pNZ5319-up-down) |
| Up-f    | CCGCTCGAGTTAATCGGTGGTTACTACTGG |
| Up-r    | CCATTATTTAATTTAAGCCGGTTTTTGC |
| Down-f  | CCGAGCTCTTGAAGACGGAGATGAGAAAC |
| Down-r  | GAGATGTCGCTCAATCTCTCCTCCCAAGT |
| For the identification of mutant strain (L. lactis N8-1) |
| Inner-f | AAATAAATGAAATGAAAGTTAA |
| Inner-r | TTCTTCTCGTGTTAGATGACT |
| Exter-f | TCGAATAAATTTGAAACCTTT |
| Exter-r | AGAACCGCTCGTAAATTTCCTG |
| Cat-f   | TCAAATAACGTTTGAATACGTT |
| Cat-r   | TACGCTCGCTCACTATTCATAT |
| Ery-f   | CTTGTCTCAATTAGTACGGTAC |
| Ery-r   | CGATAACGGTTACAAATGG |
| For the RT-qPCR |
| Q-tufA-f | GACCTCTTGGACGGAGATGAC |
| Q-tufA-r | TTCCTTACCTTTAGCACCATT |
| Q-rpsN-f | CTCGAAATTTCTCAATACAAAGC |
| Q-rpsN-r | GGCAGAGACGATAGCGAAAAGT |
| Q-rplR-f | TGTGACTAATAATGAAACGCG |
| Q-rplR-r | TGCAACAGTCCGATAGAGGGT |
| Q-csc2B-f | AACAGGAGGAGATCCCTCATC |
| Q-csc2B-r | GGTACGATCCGATCCAGTT |
| Q-csc2C-f | GCCAGTTTACACGGGACAGGTC |
| Q-csc2C-r | GGATTAGGCCTTTCGATCGCAT |
| Q-pIL399-f | TAGTGCAGGACGGAATCTT |
| Q-pIL399-r | TATCTGCAGGATTTGATGTTCT |
| Q-pp423-f | CGCAAGAAGAATGAGCGAACG |
| Q-pp423-r | GATGCGCCAGCGAGATAA |
| Q-butA-f | ATATCAACGGAACCTTACAAGC |
| Q-butA-r | TCCATCCATCATCTTCCAGC |
| Q-butB-f | CGTCAGCAGAACATCTCATACT |
| Q-butB-r | TCAGCCATCCCTCCCAACAT |
| Q-arcA-f | AAGACGGGTATCCGCCAG |
| Q-arcA-r | TTTGTGATGCTGTAGGAGAGAA |
| Q-arcB-f | GCCCTAGATTACCTTACCTC |
| Q-arcB-r | TTTCCTACTGAGATGTTCTC |
| Q-galM-f | AATGGTTGCTGGTATAGCTG |
| Q-galM-r | ATCCACAACTGATGCTGGT |
| Q-galk-f | CTCTATGGGACGCTAACCCTCA |
| Q-galk-r | TTCTACGATCCATCTCTCAG |
| Q-lacZ-f | CCGTCGCCGTCTCCTCTCATACCTT |
| Q-lacZ-r | AGTACATCCGTCCGTTG |

(Takara) and the L. lactis N8 chromosome was used as template. Then, the fragments were ligated into the XhoI–SwaI and SacI–BglII restriction sites of pNZ5319. The recombinant plasmid pNZ5319-up-down obtained was transformed into E. coli DH5α cells strain by CaCl₂
the reference and the sample are found by Integrative Genomics Viewer software (version 2.6.3; https://igv.org). The variation map of the whole genome was created by Circos (version 0.6; http://circos.ca/software) to show reads coverage and the distribution of insertion and deletion information.

Phenotype microarray analysis of the lactococcal strains (BIOLOG)

Phenotype microarray system (Biolog, California, USA) was used to determine the metabolism of wild-type and mutant strains with GP2 MicroPlate™ [4]. Sample preparation and assays were conducted following the manufacturer’s instructions. Briefly, a sterilized cotton swab was used to collect lactococcal cells from the surface of the solid medium and the cells were suspended in the inoculation (0.40% sodium chloride, 0.03% Pluronic F-68, and 0.02% Gellan Gum) (Biolog). The cell density of different strains was equalized, and 150 μL of the samples were pipetted into GP2 plate with various substrates. The plates were sealed with a film and incubated in the OmniLog® instrument (Biolog) at 30 °C for 24 h. The data were recorded automatically by the machine every 30 min (15 s of shaking before recording), and analyzed using OL-OM 3.0 software (Biolog).

Assessment of growth profiles

For growth profile experiments, L. lactis strains were cultured at 30 °C for 6 h in static (non-aerated) condition to log phase, harvested by centrifugation (5000×g, 3 min), and washed twice with PBS (phosphate buffered saline, pH 7.4). After washing, the cells were resuspended (adjusted to the same initial cell concentration) in GM17 medium as seed. For the determination of growth curves at different temperatures, we transferred 200 μL of each sample to a Bioscreen honeycomb plate (100 wells). The growth profiles were monitored by measuring OD600 for 18 h at different temperatures (30 °C, 37 °C, 39 °C) by using the Bioscreen C™ system (Lab-systems, Helsinki, Finland) [54]. For the determination of growth curves at different nisin concentrations, standard nisin was added at various concentrations (4000, 5000, 6000, 6500, 7000, and 7500 IU/mL, respectively) to GM17 medium to obtain six groups. Then, the seeds were inoculated at a concentration of 1% and 200 μL of each sample were transferred to a Bioscreen honeycomb plate (100 wells). The growth profiles were monitored by measuring OD600 for 48 h at 30 °C by using the Bioscreen C™ system. For the determination of growth curves at different lactic acid concentrations, lactic acid [80% (v/v)] was added at various concentrations [0.24% (v/v), 0.32% (v/v), and, 0.40% (v/v), respectively] to GM17 medium to obtain three groups. Then, the seeds were inoculated at a concentration of 1% and 200 μL of each sample were transferred to a Bioscreen honeycomb plate (100 wells). The growth profiles were monitored by measuring OD600 for 18 h at 30 °C by using the Bioscreen C™ system. The OD600 was measured at 15-min intervals, and static culture was gently agitated for 10 s before each measurement.

Assessment of nisin immunity

The nisin immunity of wild-type and mutant strains was determined by the 96-well plate gradient dilution method [25] with minor modifications. Briefly, the wild-type and mutant strains were grown for 6 h in antibiotic-free GM17 medium, and then washed twice with PBS. After washing, the cells were resuspended in GM17 medium and adjusted to the same cell concentration, and then diluted to a ratio of 1:1000 as seed solution. Nisin was added with a gradient concentration of 2000–8000 IU/mL at intervals of 500 IU/mL. Subsequently, 2 μL of seed solution were added into 198 μL of gradient nisin medium, and the mixtures were incubated at 30 °C for 20 h in 96-well plate. The cell concentration data were collected by microplate reader (Synergy 2, BioTek Instruments, Inc.) at a wavelength of 600 nm. The nisin immunity of the strains was determined as the minimum nisin concentration needed to ensure that the turbidity did not change to >10% at 600 nm. The next lower nisin concentration was determined as the nisin immunity level of the tested strain.

Assessment of lysozyme and lactic acid tolerances

To determine lysozyme and lactic acid tolerances, the lactococcal cells were cultured at 30 °C for 6 h in static (non-aerated) condition to log phase and harvested by centrifugation (5000×g, 3 min), washed twice with PBS, and resuspended in equal volume of GM17 medium. Lysozyme and lactic acid were added to the cell suspension at a final concentration of 10 mg/mL and 1.5% (v/v), respectively. Cell viability was determined at various time points (30, 60, 90, and 120 min) by colony counting. A total of 5 μL of serially diluted cell suspension were spotted on GM17 plates and cultured at 30 °C for 20 h, and then the plates were photographed. For survival rate experiments, colonies on plates containing 20–200 CFU were counted [44].

Assessment of nisin yield

Nisin yield was determined by the agar well diffusion method [55] with minor modifications. Briefly, the broth of the tested strains after fermentation was boiled for 10 min and cells were removed by centrifugation at 8000 rpm for 3 min. Then, the supernatant was appropriately diluted with 0.02 M HCl. M. luteus (10⁷ CFU/mL) was used as indicator and inoculated at a concentration
of 1% (v/v) into 30-mL melted/cooled LB agar. To enhance nisin diffusion, 1.5% (v/v) Tween 80 (JiangTian, Tianjin, China) was added to the medium and mixed well. Then, the medium was quickly poured into sterile plates. After solidification and pre-cultivation, a 7-mm-diameter sterile cork borer (MRS Scientific Ltd, Wickford, UK) was used to generate agar well for loading samples. Standard nisin solutions (concentrations of 20, 40, 80, 100, 200, and 400 IU/mL) were prepared using nisin powder. Subsequently, the standard nisin solutions and sample solutions were respectively loaded into the wells (80 μL per well), and the plates were incubated at 37 °C for 24 h. The diameter of inhibition zone was measured by calipers. A regression equation was derived from the nisin standard data.

SEM

The lactococcal cells were cultured at 30 °C for 6 h to exponential phase, harvested at 8000 rpm for 2 min, and washed twice with PBS. The cells were resuspended in equal volume of GM17 medium (supplemented with lysozyme to a final concentration of 17.5 mg/mL) and incubated for 1 h and 2 h. The bacterial cells without lysozyme treatment were set as controls. After incubation, the cells were washed with PBS and fixed overnight in 2.5% glutaraldehyde (Sigma) at 4 °C. Then, the cells were washed with PBS and dehydrated using gradient ethanol (50–100%, 15 min for each gradient). After dehydration, ethanol was replaced with tertiary butyl alcohol (Sigma). Subsequently, the samples were added onto the plate and prepared for SEM (QUANTA 200) observation [56].

Flasks fermentation and fed-batch fermentation

After overnight cultivation, L. lactis N8 and L. lactis N8-1 were inoculated in GM17 medium for 6 h as seed cultures. To perform the flask fermentation experiments, 250-mL Erlenmeyer flasks containing 100 mL of GM17 fermentation medium were used. A total of 1 mL of the seed cultures was inoculated into static flasks and incubated for 16 h at 30 °C. Samples were collected every 2 h for the analysis of cell density, fermentation broth pH, and nisin production. Fed-batch fermentation experiments were conducted at 30 °C for 16 h. Initially, the pH of the three groups of fermentation medium was adjusted to pH 6.0, 5.5, and 5.0 with 80% (v/v) lactic acid, respectively. Then, three groups of media were inoculated with 1% of seed culture. The pH of the three fermentation broths was controlled at 6.0, 5.5, and 5.0 by adding 10 M NaOH, respectively. The fermentation broths were sampled every 2 h for the analysis of cell density, fermentation broth pH, and nisin production.

TMT quantitative proteomics and analysis

TMT-labeled quantitative proteomics technology was applied to reveal changes in protein synthesis of L. lactis N8-1 and perform functional analysis of differentially synthetized proteins. The TMT quantitative proteomics of L. lactis N8 and L. lactis N8-1 were performed by Shanghai Majorbio Bio-pharm Technology Co., Ltd. (Shanghai, China) as following:

Total protein extraction

The L. lactis samples were suspended with protein lysis buffer (1% SDS, 200 mM DTT, 50 mM Tris–HCl, pH 8.8) which included appropriate protease inhibitor to inhibit protease activity, and treated for three times by high throughput tissue crusher Wonbio-96c (WanBo biotechnology co., Ltd, Shanghai, China) for 40 s. Then, the mixture was incubated on ice for 30 min during which the samples were vortexed 5–10 s every 5 min. Samples were incubated at 100 °C for 10 min, then transferred on ice for 30 min. After that, samples were centrifuged at 12,000×g for 20 min at 4 °C, and supernatants were collected. Five volumes of pre-cooled acetone (Sigma) were added and proteins were precipitated at –20 °C overnight. The second day, the solution was centrifuged at 12,000×g for 20 min at 4 °C. The supernatant was discarded and the precipitation was washed twice with 90% pre-cooled acetone. Lysis buffer (1% SDS, 8 M urea, cocktail) was used to resuspend the precipitation, then samples were centrifuged at 12,000×g for 20 min at 4 °C. The protein concentration in the supernatant was determined by bicinchoninic acid (BCA) method using BCA Protein Assay Kit (Beyotime biotechnology, Shanghai, China).

Protein digestion and TMT labeling

One hundred microgram of proteins were resuspended with tetraethylammonium bromide (Haibang Industry, Jinan, China) at a final concentration of 100 mM. The mixture was reduced with tris (2-carboxyethyl) phosphine (Sigma) at a final concentration of 10 mM at 37 °C for 60 min, and alkylated with iodoacetamide (Sigma) at a final concentration of 40 mM at room temperature for 40 min in darkness. Six fold volumes of cold acetone were added to precipitate protein at –20 °C for 4 h. After centrifugation at 10,000×g at 4 °C for 20 min, the pellet was resuspended with 100 μL 50 mM triethylammonium bicarbonate buffer (Sigma). Trypsin was added at 1:50 trypsin-to-protein mass ratio and incubated at 37 °C overnight. Trypsin-digested peptides were labeled with 10-plex TMT reagents (Thermo) according to the manufacturer’s instructions. Briefly, one unit of TMT reagent were thawed and reconstituted in 50 μL acetoni-trile (Sigma). After tagging for 2 h at room temperature,
hydroxylamine (Thermo) was added to react for 15 min at room temperature. Finally all samples were pooled, desalted and vacuum-dried.

High pH RPLC separation and LC–MS/MS analysis
The pooled samples were fractionated into fractions by ACQUITY Ultra Performance liquid chromatography (Waters, USA) with ACQUITY UPLC BEH C18 Column (1.7 μm, 2.1 mm × 150 mm, Waters, USA) to increase proteomic depth. Briefly, peptides were first separated with a gradient of elution (Phase B: 5 mM ammonium hydroxide solution containing 80% acetonitrile, pH 10) over 66 min at a flow rate of 200 μL/min. Twenty fractions were collected from each sample, which were subsequently pooled, resulting in ten total fractions per sample. Then, trypsin-digested peptides were analyzed by online nano flow liquid chromatography tandem mass spectrometry performed on an EASY-nLC system (Thermo Fisher Scientific) connected to a Q Exactive quadrupole orbitrap mass spectrometer (Thermo) through a nano-electrospray ion source. Briefly, the C18-reversed phase column (75 μm × 25 cm, Thermo Fisher Scientific) was equilibrated with solvent A (A: 2% ACN with 0.1% formic acid) and solvent B (B: 80% ACN with 0.1% formic acid). The peptides were eluted using the following gradient: 0–1 min, 0–5% B; 1–63 min, 5–23% B; 63–88 min, 23–48% B; 88–89 min, 48–100% B; and 89–95 min, 100% B. The tryptic peptides were separated at a flow rate of 300 nL/min. The Q Exactive plus instrument was operated in the data-dependent acquisition mode (DDA) to automatically switch between full scan MS and MS/MS acquisition. The survey of full scan MS spectra (m/z 350–1300) was acquired in the Orbitrap with 70,000 resolution. The automatic gain control (AGC) target at 1e6 and the maximum fill time was 50 ms. Then the top 20 most intense precursor ions were selected into collision cell for fragmentation by higher-energy collision dissociation (HCD). The MS/MS resolution was set at 35,000 (m/z 100), the automatic gain control (AGC) target at 1e5, the maximum fill time at 100 ms, and dynamic exclusion was 18 s.

Protein identification
MS/MS spectra were searched using Protein Discoverer™ Software 2.1 (Thermo Fisher Scientific) against Streptococcus lactis database and the decoy database as the following parameters. The highest score for a given peptide mass (best match to that predicted in the database) was used to identify parent proteins. The parameters for protein searching were set as follows: tryptic digestion with up to two missed cleavages, carbamidomethylation of cysteines and the TMT of N-terminus and lysine side chains of peptides as a fixed modification, and oxidation of methionines and protein N-terminal acetylation as variable modifications. Peptide spectral matches were validated based on Q-values at a 1% false discovery rate (FDR). Only the proteins which has at least one unique peptide was used for protein identifications. Proteins that changed more than 1.5-fold (up or down regulated) with a P-value less than 0.05 between L. lactis N8-1 and L. lactis N8 were deemed significantly difference proteins. The Uniprot database (https://www.uniprot.org/) and Pfam (http://pfam.xfam.org) were used for protein sequence search, alignment analysis, and protein domain prediction. The databases used for metabolic pathway search and analysis were iPath3.0 (https://pathways.embl.de/) and KEGG (https://www.genome.jp/kegg/). NCBI BLASTp (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) was used for protein function domain prediction. Subcellular location database (https://abi-servicess.informatik.uni-tuebingen.de/yloc/webloc.cgi) was used to predict the location where proteins appear in the cell.

Quantitative real-time PCR
Lactococcus lactis N8 and L. lactis N8-1 were cultured in GM17 medium for 6 h and appropriately diluted to achieve similar cell density. Then, the total RNA was extracted and reversed-transcribed into first-strand cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). The gene transcription levels of rpsN, rplR, csc2B, csc2C, pi339, pp423, butA, butB, arcA, arcB, galM, galK and lacZ were then assessed through RT-qPCR to confirm the proteomics data. The tufA gene was selected as housekeeping gene [57] and comparative CT(2−ΔΔCt) method was employed for data analysis. Transcription with more than two-fold change was regarded as statistically significantly different.

Statistical analysis
The experiments to determine the growth profiles of L. lactis under different treatments (nisin, temperature, and lactic acid) were performed in independent biological triplicates, and each sample was additionally collected in technical triplicates. The experiments to determine the survival rates of L. lactis under different treatments (lysozyme and lactic acid) were independently repeated at least three times. The data are shown as mean ± standard deviation (SD). Assays to determine the nisin immunity of L. lactis in 96-well plate and nisin yield of different L. lactis strains under diverse treatments (pH 6.0, 5.5, 5.0) were independently repeated at least three times. The difference between two groups was compared by the Student’s t-test and values with P<0.05 were considered significant. RT-qPCR experiments were
independently repeated at least three times, and the data are given as mean ± SD. Statistical analyses of the data were performed using Origin 85 software version 8.5.0 SRI (OriginLab Corporation, USA) and GraphPad Prism 5 software version 5.01 (GraphPad software, Inc.).

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12934-020-01487-x.

Additional file 1: Fig. S1. KEGG functional enrichment (L. lactis N8-1 vs L. lactis N8). a Upregulated proteins (> 1.2-fold change with P < 0.05); b downregulated proteins (> 1.2-fold change with P < 0.05). Fig. S2. Gene Ontology (GO) function annotations. a all proteins; b upregulated proteins (> 1.2-fold change with P < 0.05); c downregulated proteins (> 1.2-fold change with P < 0.05). Fig. S3. Diagram of protein subcellular localization. Red bars indicate upregulated proteins (> 1.2-fold change with P < 0.05), green bars indicate downregulated proteins (> 1.2-fold change with P < 0.05). Fig. S4. RT-qPCR analysis of genes with higher and lower expression level. pXN, pPfr: ribosomal protein; csc2B, csc2C: cell surface protein; ps139, pp423: bacteriophage protein; butA, butB: butanoate metabolism protein; orcA, orcB: arginine biosynthesis protein; gshA, gshK: galactose metabolism protein. (aGZ, beta-galactosidase; Fig. S5. PCR verification of the knockout vector construction. Table S1. Gene content of deleted PRF. Table S2. Substrate consumption ratio. Table S3. Nisin-immunity of engineered strains. Table S4. Upregulated proteins (> 1.2-fold change with P < 0.05). Table S5. Downregulated proteins (> 1.2-fold change with P < 0.05).

Abbreviations
LAB: Lactic acid bacteria; GTFME: Global transcription machinery engineering; PRF: Prophage-related fragment; MS: Mass spectrometry; 2-DE: Two-dimensional gel electrophoresis; TMT: Tandem mass tags; HSP: Heat shock protein; CPR: Cold shock protein; BCA: Biochinoic acid; SD: Standard deviation.

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Authors’ contributions
WQ designed the study, analyzed the primary data and drafted the manuscript. WQ, YQ, FL, Y2, and RL carried out the experiments. ZW, HX, PS, and MQ supervised the whole research work and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article and its additional file.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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References
1. Jensen PR, Liu J, Chan SHL, Chen J, Solem C. Systems biology—a guide for understanding and developing improved strains of lactic acid bacteria. Front Microbiol. 2019;10:876.
2. Song AA-L, In LL, Lim SHE, Rahim RA. A review on Lactococcus lactis: from food to factory. Microbiol Cell Fact. 2017;16:55.
3. Broadbent JR, Larsen RL, Deibel V, Steele JL. Physiological and transcriptional response of Lactobacillus casei ATCC 354 to acid stress. J Bacteriol. 2010;192:2445–58.
4. Zhu D, Fu Y, Liu F, Xu H, Saris PEJ, Qiao M. Enhanced heterologous protein productivity by genome reduction in Lactococcus lactis NZ9000. Microbiol Cell Fact. 2017;16:1.
5. Gao X, Jiang L, Zhu L, Xu Q, Xu X, Huang H. Tailoring of global transcription sigma D factor by random mutagenesis to improve Escherichia coli tolerance towards low-pHs. J Bacteriol. 2016;224:55–63.
6. Alper H, Moxley J, Nevoigt E, Fink GR, Stephanopoulos G. Engineering yeast transcription machinery for improved ethanol tolerance and production. Science. 2006;314:1566–8.
7. Gao X, Yang X, Li J, Zhang Y, Chen P, Lin Z. Engineered global regulator H-NS improves the acid tolerance of Escherichia coli. Microbiol Cell Fact. 2018;17:1118.
8. Li Y, Xu X, Qu R, Zhang G, Rajoka MSR, Shao D, Jiang C, Shi J. Heterologous expression of Deinococcus radiodurans sHSP20 confers temperature stress tolerance in Escherichia coli. Cell Stress Chaperones. 2018;23:653–62.
9. Zhang M, Zhang K, Mehmood MA, Zhao ZK, Bai F, Zhao X. Deletion of acetate transporter gene adi2 improved tolerance of Saccharomyces cerevisiae against multiple stresses and enhanced ethanol production in the presence of acetic acid. Biotechnology. 2017;245:1461–8.
10. Dong X, Tian B, Dai S, Li T, Guo L, Tan Z, Jiao Z, Jin Q, Wang Y, Hua Y. Expression of Pprf from Deinococcus radiodurans improves lactic acid production and stress tolerance in Lactococcus lactis. PLoS ONE. 2015;10:e0142918.
11. Desmond C, Fitzgerald G, Stanton C, Ross R. Improved stress tolerance of GroESL-overexpressing Lactococcus lactis and probiotic Lactobacillus paracasei NFBC 338. Appl Environ Microbiol. 2004;70:5929–36.
12. Wang Y, Li Y, Ye X, Yu L, Feng Y. Genome-shuffling improved acid tolerance and L-lactic acid volumetric productivity in Lactobacillus rhamnosus. J Biotechnol. 2007;129:510–5.
13. Qi J, Caiyin Q, Wu H, Tian K, Wang B, Li Y, Qiao J. The novel sRNA s015 improves nisin yield by increasing acid tolerance of Lactococcus lactis F44. Appl Microbiol Biotechnol. 2017;101:6483–93.
14. Li Y, Kan Z, You Y, Gao X, Wang Z, Fu R. Exogenous transglutaminase improves multiple-stress tolerance in Lactococcus lactis and other lactic acid bacteria with glutamine and lysine in the cell wall. Biotechnol Lett. 2015;37:2467–74.
15. López-González MJ, Campelo AB, Picón A, Rodríguez A, Martínez B. Resistance to bacteriocin Lcn972 improves oxygen tolerance of Lactococcus lactis IPLA947 without compromising its performance as a dairy starter. BMC Microbiol. 2018;18:176.
16. Kimoto H, Ohmomo S, Okamoto T. Enhancement of bile tolerance in lactococci by Tweep 80. J Appl Microbiol. 2002;92:41–6.
17. Liang F, Zhang Y, Xu B, Zhao L, Liu X, Gao W, Ma T, Yang C, Wang S, Liu R. Deletion of genomic islands in the Pseudomonas putida KT2440 genome can create an optimal chassis for synthetic biology applications. Microbiol Cell Fact. 2020;19:1–12.
18. Martínez-García E, Nikel P, Aparicio T, de Lorenzo V. Pseudomonas 2.0: genetic upgrading of P.putida KT2440 as an enhanced host for heterologous gene expression. Microbiol Cell Fact. 2014;13:159.
19. Zhang H, Zhang C, Wang H, Yan Y 2, Sun J. A novel prophage lysozyme PlyS218 with extended lytic activity and stability against Streptococcus suis infection. FEMS Microbiol Lett. 2016;363:fwr186.
20. Wu H, Zhao Y, Du Y, Miao S, Liu J, Li Y, Caiyun Q, Qiao J. Quantitative proteomics of Lactococcus lactis F44 under cross-stress of low pH and lactate. J Dairy Sci. 2018;101:6872–84.

21. Kramer NE, Hasper HE, van den Bogaard PT, Morath S, de Kruijff B, Hartung T, Smidt EJ, Breukink E, Kok J, Kuipers OP. Increased D-lalanation of lipoteichoic acid and a thickened septum are main determinants in the nisin resistance mechanism of Lactococcus lactis. Microbiology. 2008;154:1755–62.

22. Kelleher P, Mahony J, Schweinlin K, Neve H, Franz CM, van Sinderen D. Assessing the functionality and genetic diversity of lacticococal prophanes. Int. J Food Microbiol. 2018;272:29–40.

23. Acouturier A, Chain F, Langella P, Bidnenko E. Characterization of a prophyage-free derivative strain of Lactococcus lactis ssp. lactis IL1403 reveals the importance of prophanes for phenotypic plasticity of the host. Front Microbiol. 2018;9:2032.

24. Kim W, Hall R, Dunn N. Improving nisin production by increasing nisin immunity/resistance genes in the producer organism Lactococcus lactis. Appl Microbiol Biotechnol. 1998;50:429–33.

25. Xuanyuan Z, Wu Z, Li R, Jiang D, Su J, Xu H, Bai Y, Zhang X, Saris PEJ, Qiao M. Loss of HipF function in Lactococcus lactis subsp. lactis N8 results in increased nisin resistance. Curr Microbiol. 2010;61:329–34.

26. Sun Z, Zhong J, Liang X, Liu J, Chen X, Huan L. Novel mechanism for nisin resistance via proteolytic degradation of nisin by the nisin resistance protein NSR. Antimicrob Agents Chemother. 2009;53:1964–73.

27. Qiao M, Immonen T, Koponen O, Saris PE. The cellular location and effect on nisin immunity of the Nsl protein from Lactococcus lactis NB8 expressed in Escherichia coli and L. lactis. FEMS Microbiol Lett. 1995;131:75–80.

28. Wilkins MR, Sanchez J-C, Gooley AA, Appel RD, Humphrey-Smith I, Hochstassler DF, Williams KL. Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it. Biotechnol Genet Rev. 1996;13:19–50.

29. Ong S-E, Mann M. Mass spectrometry-based proteomics turns quantitative. Nat Chem Biol. 2005;1:252–62.

30. Aebersold R, Mann M. Mass spectrometry-based proteomics. Nature. 2003;422:198–207.

31. Lambert JM, Bongers RS, Kleerebezem M. Cre-based system for multiple-gene deletions and selectable-marker removal in Lactococcus lactis subsp. lactis N8. FEMS Microbiol Lett. 1998;64:15–21.

32. Papadimitriou K, Alegría Á, Bron PA, de Angelis M, Gobbetti M, Kleerebezem M. Co-expression of nisin Z and Leucocin C as a basis for effective protection against Lactococcus lactis and other gram-positive bacteria. J Biotechnol. 2015;65:1659–65.

33. Saris PEJ, Akçelik M. Influence of growth conditions on the production of bioengineered Lactococcus lactis strains. J Ind Microbiol Biotechnol. 2009;36:481.

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20. Wu H, Zhao Y, Du Y, Miao S, Liu J, Li Y, Caiyun Q, Qiao J. Quantitative proteomics of Lactococcus lactis F44 under cross-stress of low pH and lactate. J Dairy Sci. 2018;101:6872–84.

21. Kramer NE, Hasper HE, van den Bogaard PT, Morath S, de Kruijff B, Hartung T, Smidt EJ, Breukink E, Kok J, Kuipers OP. Increased D-lalanation of lipoteichoic acid and a thickened septum are main determinants in the nisin resistance mechanism of Lactococcus lactis. Microbiology. 2008;154:1755–62.

22. Kelleher P, Mahony J, Schweinlin K, Neve H, Franz CM, van Sinderen D. Assessing the functionality and genetic diversity of lacticococal prophanes. Int. J Food Microbiol. 2018;272:29–40.

23. Acouturier A, Chain F, Langella P, Bidnenko E. Characterization of a prophyage-free derivative strain of Lactococcus lactis ssp. lactis IL1403 reveals the importance of prophanes for phenotypic plasticity of the host. Front Microbiol. 2018;9:2032.

24. Kim W, Hall R, Dunn N. Improving nisin production by increasing nisin immunity/resistance genes in the producer organism Lactococcus lactis. Appl Microbiol Biotechnol. 1998;50:429–33.

25. Xuanyuan Z, Wu Z, Li R, Jiang D, Su J, Xu H, Bai Y, Zhang X, Saris PEJ, Qiao M. Loss of HipF function in Lactococcus lactis subsp. lactis N8 results in increased nisin resistance. Curr Microbiol. 2010;61:329–34.

26. Sun Z, Zhong J, Liang X, Liu J, Chen X, Huan L. Novel mechanism for nisin resistance via proteolytic degradation of nisin by the nisin resistance protein NSR. Antimicrob Agents Chemother. 2009;53:1964–73.

27. Qiao M, Immonen T, Koponen O, Saris PE. The cellular location and effect on nisin immunity of the Nsl protein from Lactococcus lactis NB8 expressed in Escherichia coli and L. lactis. FEMS Microbiol Lett. 1995;131:75–80.

28. Wilkins MR, Sanchez J-C, Gooley AA, Appel RD, Humphrey-Smith I, Hochstassler DF, Williams KL. Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it. Biotechnol Genet Rev. 1996;13:19–50.

29. Ong S-E, Mann M. Mass spectrometry-based proteomics turns quantitative. Nat Chem Biol. 2005;1:252–62.

30. Aebersold R, Mann M. Mass spectrometry-based proteomics. Nature. 2003;422:198–207.

31. Lambert JM, Bongers RS, Kleerebezem M. Cre-lox-based system for multiple-gene deletions and selectable-marker removal in Lactococcus plantarum. Appl Environ Microbiol. 2007;73:1126–35.

32. Zhou Y, Liang Y, Lynch KH, Dennis JJ, Wishart DS. PHAST: a fast phylogene search tool. Nucleic Acids Res. 2011;39:W547–52.

33. Sanders JW, Venema G, Kok J. Environmental stress responses in Lactococcus lactis. FEMS Microbiol Rev. 1999;23:483–501.

34. Papadimitriou K, Alegría A, Bron PA, de Angelis M, Gobbetti M, Kleerebezem M, Lemos JA, Linares DM, Ross P, Stanton C, et al. Stress physiology and other gram-positive bacteria. J Bacteriol. 1978;133:1126–9.

35. Qiao M, Immonen T, Koponen O, Saris PE. The cellular location and effect on nisin immunity of the Nsl protein from Lactococcus lactis NB8 expressed in Escherichia coli and L. lactis. FEMS Microbiol Lett. 1995;131:75–80.

36. Wilkins MR, Sanchez J-C, Gooley AA, Appel RD, Humphrey-Smith I, Hochstassler DF, Williams KL. Progress with proteome projects: why all proteins expressed by a genome should be identified and how to do it. Biotechnol Genet Rev. 1996;13:19–50.

37. Saris PEJ, Akçelik M. Influence of growth conditions on the production of bioengineered Lactococcus lactis strains. J Ind Microbiol Biotechnol. 2009;36:481.

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