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Loss of IrpT Function in *Lactococcus lactis* subsp. *lactis* N8 Results in Increased Nisin Resistance

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Abstract The antibiotic nisin, produced by *Lactococcus lactis* subsp. *lactis* N8, offers an extensive commercial prospect as natural food preservatives. The nisin immunity of the *L. lactis* strains is regulated by a variety of mechanisms. In this study, we isolated a *L. lactis* L31 strain with increased nisin resistance from a mini-Mu transposon mutant pool of strain N8. The single Mu insertion in strain L31 was in the *irpT* gene with unknown function. By comparing the proteomic profiles of *L. lactis* L31 and its parental strain, we found that changes occurred in the synthesis of a protein involved in cell wall biosynthesis (RmlD). Strain L31 had 13.7% higher content of rhamnose in the cell wall than the N8 strain. Overexpression of RmlD involved in the synthesis of dTDP-L-rhamnose in the nisin-sensitive MG1363 strain increased nisin resistance of the strain. The results indicate that these cellular proteins effected nisin resistance in *L. lactis* N8.

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Introduction

Nisin is a post-translationally modified lanthionine-containing peptide antibiotic, which is produced by certain strains of *Lactococcus lactis* and *Streptococcus uberis* [1, 2]. In order to avoid self-destruction, the nisin-producing *L. lactis* strains protect themselves against the bactericidal activity of nisin, which is called nisin immunity [3]. Full nisin immunity requires four polypeptides, the membrane-bound lipoprotein NisI, an ABC transporter complex containing a cytoplasmic NisF, and two integral membrane proteins, NisE and NisG [4, 5]. Functional analysis of these four components showed that protection mediated by NisI occurs at the outer side of the cytoplasmic membrane, the location of lipoproteins, and seems to function partly by intercepting nisin. In addition, part of the translocated NisI escapes lipid modification and is secreted to the medium contributing to nisin immunity. NisI seems to cooperate with the ABC type transporter NisFEG that exports nisin from the cell surfaces into the medium.

Non-nisin-producing strains can develop nisin resistance. For example, through stepwise exposure of cells to increasing concentrations of nisin, the generation of nisin-resistant (Nis^r) strains of *Listeria. monocytogenes* and *L. lactis* was achieved [6, 7]. Through genetic engineering of *Bacillus subtilis*, nisin resistance was increased [8]. Transcriptome analysis of nisin-resistant strains revealed many differentially expressed genes involved in nisin resistance [9]. So far, five major nisin resistance mechanisms could be concluded from these analyses. The first mechanism is the prevention of nisin from reaching the membrane and the lipid II molecule [10]. Second, other *L. lactis* Nis^r strains could change the local pH at the outer surface of the cytoplasmic membrane. Third, acquiring nisin resistance may come from changing the elongation and saturation of

phospholipids. Fourth, ABC transporters might be involved in expelling nisin from the cytoplasmic membrane, preventing nisin–lipid II binding and pores forming. Finally, expression of nisin-inactivating enzymes could protect strains [11].

Since nisin immunity/resistance is a complex system, several challenges still exist in understanding this system [3]. In this study, through mini-Mu transposon technique, a gene with unknown function, named *irpT* in *L. lactis* N8 genome, was found to increase nisin resistance. Proteome analysis of the *irpT* mutant identified several proteins potentially responsible for this resistance increase. One of these, RmlD involved in the synthesis of dTDP-L-rhamnose and needed for the biosynthesis of the cell wall, mediated nisin resistance upon overexpression in a nisin-sensitive *L. lactis* strain.

Materials and Methods

Strains and Plasmids

All bacterial strains and plasmids used in this study are presented in Table 1. The *Lactococcus lactis* strains were grown at 30°C without agitation in M17 medium (Oxoid, Hampshire, UK) supplemented with 0.5% glucose and 0.5% sucrose (M17GS). For trans-complementation, the lactococcal expression plasmid pLEV16 was constructed by PCR amplification, which included in the pWV01 replicon, the constitutive P45 promoter, the chloramphenicol resistance gene of pSG13 and the kanamycin resistance gene of pHTH2. The complete *irpT* gene PCR product was cloned into the plasmid pLEV16, downstream from the promoter P45, yielding pLEV17. The plasmid pLEV16 carried a 1,039 bp *rmlD* gene fragment, yielding pLEV18.

Mini-Mu Transposon Technique and Southern Blotting

Lactococcus lactis N8 was used as a target bacterium for the Mu insertion mutagenesis. The methods for preparation of electrocompetent cells, gene cloning, and sequencing were described previously [12]. Using the mini-Mu transposition technique in *L. lactis* N8, we generated a genomic random insertion mutant library of approximately 1,800 member clones. Genomic DNA, digested with *Pvu*II and *Eco*RI, respectively, was electrophoresed on a 1% agarose gel and blotted onto a nylon membrane. Southern hybridization was carried out with DIG-High Prime labeled (Roche, Mannheim, Germany) Em-Mu was used as a probe. Restriction enzymes and Taq DNA polymerases were purchased from TaKaRa (Dalian, China).

Determination of Minimum Inhibitory Concentrations

The insertion mutant pool was screened for altered minimum inhibitory concentrations of nisin, which were estimated from the growth curves similarly as in Takala and Saris [13]. The *L. lactis* strains were grown overnight in antibiotic-free M17GS medium, diluted 1:100, and nisin was added to concentrations of 0–7,000 IU ml^{-1} for screening the ones with changed MIC of nisin. The MIC was determined as the minimum nisin needed to ensure that the culture did not grow to over 10% of the relative cell density at 600 nm in 16 h. *L. lactis* L31 was identified from the mutant pool that portrayed an increased Nisin resistance. Nisin standard sample was purchased from Sigma (Steinheim, Germany).

Two-Dimensional Gel Electrophoresis

For two-dimensional gel electrophoresis (2-DE) analysis, 50 ml of the lactococcal culture was harvested in the logarithmic period of growth by centrifugation. The cell pellets were suspended in 3 ml of lysis buffer (7.0 mol l^{-1} urea, 2.0 mol l^{-1} thiourea, 4% CHAPS). The cells were disrupted with Scientz JY92-II sonifier (Ningbo, China). 2-DE was carried out as described by Görg et al. [14] using the 2-DE system from Bio-Rad (Hercules, CA, USA). The first dimension (IEF) was performed using a 17 cm IPG strips with a linear gradient ranging from 4 to 7. The second dimension was carried out on 12% polyacrylamide

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference/Source
Strains		
Lactococcus lactis N8	Nisin Z producer	[1]
L. lactis L31	*L. lactis* N8::Mu mutant	This study
L. lactis MG1363	Plasmid free, highly nisin-sensitive (Nis^s)	[21]
Plasmids		
pLEV16	Expression vector with P45, Kan^r, Cm^r	This study
pLEV17	pLEV16 carrying a 899 bp *irpT* gene fragment from *L. lactis* N8, Kan^r, Cm^r	This study
pLEV18	pLEV16 carrying a 1039 bp *rmlD* gene fragment from *L. lactis* N8, Kan^r, Cm^r	This study

gels. PDQuest Analysis Software was used for image analysis.

Real-Time Quantitative PCR

All primer pairs were designed based on the corresponding gene sequence of *L. lactis* SK11 [15]. Total RNA of *L. lactis* strains was extracted with TRIzol Reagent from Invitrogen (Carlsbad, CA, USA), according to the manufacturer's protocol. RT-qPCR was performed on the iQ5 real-time system (Bio-Rad, Hercules, CA, USA), using *TranStart* Green qPCR SuperMix (TransGen, Beijing, China). All RT-qPCR reactions were repeated independently three times and each repeat contained three biological replicates. Data analysis was carried out using the comparative C_T ($2^{-\Delta\Delta CT}$) method with 16S rRNA as the internal reference gene [16].

Isolation and Characterization of Cell Wall Sugars

Isolation of cell wall sugars was performed as described by Looijesteijn et al. [17]. The monomeric sugar composition after hydrolysis was determined by high-performance liquid chromatography [18]. The values presented below are averages based on at least three independent experiments.

Results

Isolation and Phenotypic Characterization of *L. lactis* L31

Through the MIC determination of *L. lactis* N8 strain and its derivatives, a mutant strain with improved nisin resistance was isolated and named L31. The result showed that L31 with a Mu transposon had significantly higher nisin resistance than N8 (Table 2).

Identification and Sequence Analysis of Hypothetical *irpT* Gene

The flanking region of the Mu insertion site in *L. lactis* L31 was cloned and sequenced, which revealed that the disrupted gene was *irpT* in *L. lactis* N8. The *irpT* gene (708 bp) with unknown function shares 91% identity with a gene encoding a hypothetical protein in *L. lactis* SK11 [15]. The complete nucleotide sequence of the *irpT* gene has been submitted to GenBank databases, and has been assigned the accession number GQ386851.

Southern blotting was performed to investigate whether the *L. lactis* L31 had only one Mu transposon DNA copy in the genomic DNA. No signal was obtained from the control genome of the parental strain N8, whereas the genome of the *L. lactis* L31 had only one hybridization band (data not shown). This result showed that only a single copy of Em-Mu transposon DNA had inserted into the genomic DNA of *L. lactis* L31.

Table 2 MICs for nisin of various *L. lactis* derivatives used in this study

Strain	MIC (IU mL^{-1})[a]
L. lactis N8	6,000[b]
L. lactis L31	7,000
L. lactis L31–pLEV16[c]	7,000
L. lactis L31–pLEV17[c]	6,000
L. lactis MG1363	10
L. lactis MG1363–pLEV16[c]	10
L. lactis MG1363–pLEV18[c]	20

[a] MIC was expressed as the minimum inhibitory concentration (IU mL^{-1}) of nisin

[b] Each value represents the mean of at least three independent determinations

[c] The strains were made in this study

Complementation of the Inactive *irpT* Gene in Strain L31

The vector pLEV17 as well as the empty vector pLEV16 was electroporated into the *irpT* mutant strain L31, generating the strain L31-pLEV17, and the control strain L31-pLEV16, respectively. The resulting strains were grown in antibiotic-free medium and tested for MIC of nisin. The assays showed that nisin resistance of the L31 mutant was restored to that of the parent strain N8 when exogenous *irpT* was expressed in it, which further confirmed the relationship between *irpT* and nisin resistance.

Changes in the Proteome of *L. lactis* L31

In order to investigate the effects of the *irpT* gene disruption, two-dimensional polyacrylamide gel electrophoresis (2-DE) was used to compare the proteomic profiles of *L. lactis* L31 and *L. lactis* N8. The number of distinct spots detected for *L. lactis* L31 was highly similar to that found for the parental strain N8 (approx. 390). However, five spots from these two strains showed about 6-fold difference in intensity. The five proteins are dTDP-L-rhamnose synthase (RmlD), phosphocarrier protein Hpr (PtsH), phosphopyruvate hydratase (Eno), glyceraldehyde 3-phosphate dehydrogenase (GapB), and hypothetical protein (YahB), respectively. One of them (PtsH) was considered to be decreased in intensity in *L. lactis* L31, and the four other spots were increased (Eno, RmlD, YahB, and GapB; Fig. 1a, b).

In order to verify that the expression of these five genes was changed in *L. lactis* L31, the RT-qPCR was done to detect the mRNA levels of these genes. The analysis showed that the *ptsH* gene was expressed to a lower extent in *L. lactis* L31, and the *eno*, *rmlD*, *yahB*, and *gapB* genes showed that higher expression in L31 than in N8 (filled bars in Fig. 1c). Moreover, the mRNA levels were regulated in opposite directions in the complementary strain L31-pLEV17 (open bars in Fig. 1c), in which the *ptsH* gene was expressed to a higher extent, and other four genes showed that lower expression than in L31. The results of RT-qPCR confirmed up- and down-regulation in the transcription level of five genes, which were consistent with their changes shown in 2-DE.

Influence of the *irpT* Gene Mutant on Cell Wall Polysaccharides

The proteins with altered expression levels in L31 belong to three functional groups, cell wall biosynthesis, central and energy metabolism, and stress-related proteins. Among them, the *rmlD* gene is involved in the synthesis of dTDP-L-rhamnose, which is an immediate precursor for cell wall polysaccharides backbone production [19]. Cell wall polysaccharides may act as a barrier that prevents some antibiotics from reaching their target molecules in the cell wall and may partly contribute to the resistance to some antibiotics [20]. Therefore, we focused on the *rmlD* gene to investigate if this gene can influence cell wall polysaccharides and nisin resistance.

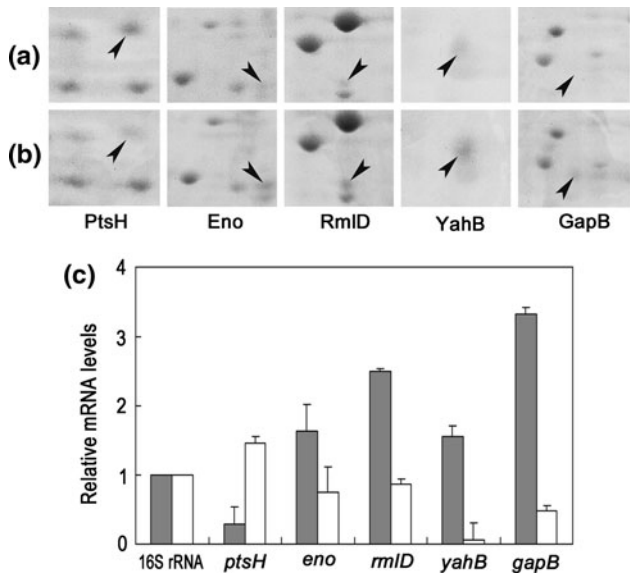

Fig. 1 Comparison of the protein pattern of *L. lactis* strains by 2-DE (pH gradient 4–7) and the mRNA level by real-time quantitative PCR. Subsection views of differentially expressed proteins on the 2-D gels of *L. lactis* N8 (**a**) and *L. lactis* L31 (**b**); (**c**) The mRNA levels of five genes in *L. lactis* L31 (*filled bars*) and strain L31-pLEV17 (chromosomal non-functional *irpT* gene and functional *irpT* in plasmid; *open bars*) relative to their corresponding genes in N8 and L31-pLEV16, respectively. The gene 16S rRNA was used as the internal reference gene. All real-time quantitative PCR results were expressed relative to 16S rRNA. The error bars represent the standard deviations based on three experiments

In order to test if the cell wall polysaccharides produced by strain L31 were changed, its sugar composition was analyzed by high-performance liquid chromatography. The result showed that the rhamnose content of cell wall polysaccharides was increased by 13.7% ($P < 0.05$) in *L. lactis* L31 compared with its parental strain. The result is consistent with the up-regulation of the *rmlD* gene expression level.

In order to investigate the relationship between nisin resistance and *rmlD* gene, RmlD was overexpressed in *L. lactis* MG1363 [21], followed by analysis of nisin resistance. The result demonstrated that RmlD overexpression improves the nisin resistance (Table 2).

Discussion

In this study, we identified and sequenced the *irpT* gene that upon inactivation increased nisin resistance of *L. lactis* N8. In the genome of *L. lactis* SK11, homologous gene of *irpT* is present at the penultimate position within the same direction of nine genes, which may generate a gene cluster. The downstream gene of *irpT* is *xerS*, encoding a site-specific tyrosine recombinase XerS [22]. Sequence analysis showed that the *xerS* gene has its own promoter, therefore the disruption of *irpT* gene may not affect *xerS* gene expression, which was also confirmed by complementation analysis and RT-PCR (data not shown).

The IrpT protein of unknown function is a membrane protein predicted by the online software SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/). In order to determine whether the IrpT protein is directly involved in promoting nisin binding to the cell surface, we performed the nisin adsorption experiment and found that loss of IrpT function in *L. lactis* L31 does not affect nisin binding to the cell surface (data not shown). Moreover, to find out whether the improvement of nisin resistance caused by *irpT* disruption is through affecting the level of membrane-bound lipoprotein NisI, we detected the amount of lipoprotein NisI. Western blot analysis showed that the disruption of *irpT* did not change the level of the lipoprotein NisI (data not shown). As the nisin immunity genes (*nisI* and *NisFEG*) are all in the same regulon the effect of *irpT* knockout seems not be a result of influence on regulation of the nisin operons.

In order to study the mechanisms involved in the increased nisin resistance of L31, we compared the proteomic profiles of *L. lactis* L31 and its parental strain N8 by

2-DE. The results showed that difference in the expression levels of five proteins, which are not present in the gene cluster of *irpT*. Four (Hpr, Eno, YahB, and GapB) of the five proteins have been investigated by previous studies to show indirect influence on nisin resistance [9]. For example, YahB is a hypothetical protein, which was defined as a universal stress protein, a UspA-like nucleotide-binding protein. Multiple members of the UspA family are found in a variety of organisms, such as bacteria, archaea, fungi, protozoa, and plants, and their synthesis is induced in response to a large number of stress conditions, including starvation for carbon, nitrogen, phosphate, sulfate, and amino acid and exposure to heat, oxidants, metals, uncouplers of the electron transport chain, polymyxin, cycloserine, ethanol, and antibiotics [23, 24]. Nisin is an antibiotic, a kind of stress for the nisin-producing *L. lactis* strains. Perhaps, YahB is contributing to the increased nisin resistance in *L. lactis* L31.

Several recent reports have suggested that the composition of the cell wall can directly influence nisin resistance [10]. For this reason, we focused on the rhamnose content of cell wall polysaccharides to investigate how the *rmlD* gene, which is overexpressed in *L. lactis* L31, influences nisin resistance. The *rml* operon is composed of four genes, *rmlABCD*, encoding glucose-1-phosphate thymidylyltransferase, dTDP-glucose-4,6-dehydratase, dTDP-4-keto-6-deoxyglucose-3,5-epimerase and dTDP-L-rhamnose synthase, respectively. Through a four-step enzymic reaction, glucose-1-phosphate turns into dTDP-L-rhamnose [25], which is a precursor of cell wall polysaccharides backbone in *L. lactis*. It has been reported that cell wall polysaccharides was absent in the cell wall of a *rmlD* mutant strain, which exhibited about 5-fold-higher sensitivity to nisin than wild strain [20], suggesting that the presence of cell wall polysaccharides may confer resistance to nisin in *Streptococcus mutans*. Although, there are many differences between streptococci and lactococci in the metabolic regulation [26], the involvement of the *rmlD* gene in *S. mutans* nisin resistance suggests that the corresponding gene in *L. lactis* may also influence nisin resistance in a similar way. Over-expression of RmlD, which is the case in L31, may increase nisin resistance potentially via the rhamnose content of the cell wall. Thus, we analyzed the sugar composition of cell wall polysaccharides in *L. lactis* L31 and *L. lactis* N8. Indeed, the rhamnose content was increased in *L. lactis* L31. Moreover, RmlD overexpression in nisin-sensitive *L. lactis* MG1363 increased the resistance level to nisin. These results implied that the disruption of the *irpT* gene may increase the nisin resistance level partially by up-regulation of RmlD protein expression.

In conclusion, nisin immunity/resistance in the nisin-producing *L. lactis* strains is comprehensively regulated by a variety of mechanisms. In this study, we discovered a novel gene *irpT*, whose disruption increased nisin resistance. IrpT did not seem to be directly involved in nisin resistance. Rather it has a regulatory role as its lack of function resulted in up-regulation of the *rmlD* gene. These results may be useful for constructing strains with increased production of nisin as the nisin resistance level is one factor restricting the concentration of produced nisin.

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