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Nisin-selectable food-grade secretion vector for *Lactococcus lactis*

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Abstract A nisin-resistant *Lactococcus lactis* strain TML01 was isolated from crude milk. A gene with 99% homology to the nisin-resistance gene, *nsr*, was identified. The food-grade secretion plasmid, pLEB690 (3746 bp), was constructed based on this novel *nsr* gene enabling primary selection with up to 5 µg nisin/ml. The functionality of pLEB690 as a secretion vector was shown by expressing and secreting the pediocin AcH gene *papA* in *L. lactis*. pLEB690 is therefore a functional food-grade secretion vector potentially useful for the food industry.

Keywords food-grade plasmid, pediocin, nisin selection

Introduction

Lactococcus lactis is widely used in dairy fermentations. A set of genetic tools has been developed to improve the natural properties of *L. lactis* (Mierau & Kleerebezem, 2005). Potential tools for genetic engineering of food bacteria are the food-grade plasmid vectors (Reviriego et al., 2007a; Takala & Saris, 2002). The DNA of these vectors is derived from strains, which are generally regarded as safe (GRAS). In addition, these vectors do not rely on antibiotics for their selection. Some vectors endow the host strains with new properties, such as nisin- cadmium- or copper-resistance (Liu et al., 2005; Takala & Saris, 2002). Some vectors adopt sugar utilization or auxotrophic supplement for selection (Platteeuw et al., 1996; Sørensen et al., 2000). These complementation vectors require host strains with special genotypes, thus limiting their usability. An ideal food-grade vector would be small, stable, allow strong selection, and contain elements for intra- or extracellular protein production.

Nisin is the only bacteriocin used as a food preservative and is therefore acceptable as a selective agent upon transformation of food-grade plasmids. The nisin immunity gene, *nisI*, from a nisin-producing *L. lactis* strain has been exploited as a selection marker in a food-grade vector (Takala & Saris, 2002). Some non-nisin-producing *L. lactis* strains carry the nisin-resistance gene *nsr* (von Wright et al., 1990), which encodes a protease that digests nisin at the MeLan²⁸ site, resulting in 100-fold reduction of nisin activity (Sun et al., 2009). The *nsr* gene has also been used as a selection marker in food-grade vectors (Froseth & McKay, 1991; Liu et al., 2005; von Wright et al., 1990). Even though the *nisI* and *nsr* vectors make cells resistant against $\geq 5 \mu\text{g}$ nisin/ml, efficient primary selection of plasmid transformants after electroporation has only been possible with lower nisin concentrations (Takala & Saris, 2002; von Wright et al, 1990). Other issues limiting the applicability of nisin-selectable vectors have been the lack of inducible promoters and secretion signals for induced and extracellular production of proteins.

In this work, a novel nisin-resistance gene *nsr* was identified and cloned from the nisin resistant *L. lactis* strain TML01. The *nsr* gene was used to construct food-grade vectors for constitutive and inducible gene expression, as well as for secretion and intracellular protein production. The new *nsr*-vectors presented here mediated higher nisin-resistance than previously reported food-grade vectors.

Materials and methods

Bacteria, plasmids and culture media

Lactococcus lactis NZ9000 (Kuipers et al., 1997), *L. lactis* N8 (Graeffe et al., 1991), and pediocin-sensitive strain *Listeria monocytogenes* WSLC 1018 (gift from Prof Martin Loessner, ETH Zürich, Switzerland) were cultivated at 30°C in M17GS medium (M17 broth supplemented with 0.5% w/v glucose and 0.55% w/v sucrose). Pediocin-producer *Lactobacillus plantarum* WHE 92 (Ennahar et al., 1996) was cultivated at 30°C in MRS broth. M17GST medium (M17GS with 1% v/v Tween80) supplemented with nisin was used for isolation of nisin-resistant strains and detection of pediocin in agar diffusion plates. The plasmid pLEB590 (Takala & Saris, 2002) was used as a template in construction of the food-grade vectors.

DNA manipulations

PCR primers used in this study are listed in the Supplementary Table 1. PCR products were purified with the EZNA Cycle-pure kit (Omega Bio-tek) and sequenced (Institute of Biotechnology, Helsinki, Finland). Plasmids were isolated with the Plasmid Mini Kit (Omega Bio-tek) with following modifications: lysozyme was used at 1 mg/ml in solution I followed by incubation at 37°C for 1 h. Electrotransformation was performed as described previously (Wu et al., 2009).

Strain isolation, identification, and nisin-resistance gene amplification

Raw bovine milk from a local farm was serially 10-fold diluted. Aliquots were spread on M17GST agar plates containing 5 µg nisin/ml and 1% (v/v) Bromocresol Purple as pH indicator. The plates were incubated overnight at 30°C. The white round colonies which changed the medium from purple to yellow were transferred to new M17GST agar plates supplemented with 12.5 µg nisin/ml. The 16S rRNA gene of the isolated strain was amplified and sequenced using the primers 16S1 and 16S2.

Primers *nsr1* and *nsr2* were used to amplify the nisin-resistance gene *nsr* using the isolated strain as template. PCR was performed using the Dynazyme DNA polymerase (Finnzymes) under the conditions of: 95°C for 2 min; 30 cycles of 94 °C for 20 s, 40 °C for 15 s, and 72 °C for 1 min; and finally 72 °C for 10 min. PCR products were analysed in 1% agarose gel with 1 kb DNA ladder (Fermentas) as the size marker.

Construction of food-grade vectors

The gene, *nsr*, was amplified by PCR using Phusion DNA polymerase (Finnzymes) with primers *nsr3* and *nsr4*, digested with *HindIII* and *EcoRV*, and ligated with a *HindIII-EcoRV* -fragment from pLEB590 containing the replicon and P45 promoter. The formed food-grade vector, named pLEB688, was digested with *HindIII* and *BglII*, and ligated with the *nisZ* promoter amplified from *L. lactis* N8 with primers *PnisZ1* and *PnisZ2* (digested with *HindIII* and *BglII*). The constructed nisin-inducible, food-grade vector pLEB689 was used as a template for construction of the food-grade secretion vector pLEB690 by overlap PCR. In the first step PCR, primers *usp1* and *usp2* were used to amplify *usp45* signal sequence from NZ9000, while *nsr5* and *nsr6* were used to amplify pLEB689. The first step products were used as templates in the second step PCR with the primers *nsr5* and *usp2*. Finally, the second step product was restricted with *BglII* and self-ligated.

Analysis of nisin-resistance level and plasmid stability

L. lactis NZ9000 was electrotransformed with the constructed plasmids. Nisin-resistance was determined with a plate reader at 30°C for 20 h with wide band filter of 420-580 nm, by inoculating 3 µl overnight bacterial culture into 300 µl M17GS medium supplemented with nisin (0 to 12.5 µg/ml) in 100-well plates. Of each sample, three parallels were made. Plasmid stability was determined essentially as described before (Platteeuw et al., 1996).

Pediocin secretion vector and pediocin bioassay

The pediocin gene *papA* without secretion signal was amplified by PCR with primers *ped1* and *ped2* using *Lb. plantarum* WHE 92 cells as template. The PCR product was digested with *BglII* and linked to the *NaeI* and *BglII* digested pLEB690 plasmid. The formed pediocin secretion vector pLEB691 (Fig. 1d) was introduced into *L. lactis* NZ9000.

The antimicrobial activity of the pediocin produced by the NZ9000/pLEB691 strain was tested by agar diffusion bioassay (Reviriego et al., 2007a) with *L. monocytogenes* WSLC 1018 as the indicator organism. *L. lactis* N8 and *Lb. plantarum* WHE 92 were used as positive controls for antimicrobial activity. NZ9000 harboring plasmid pLEB690 (no *papA* gene) was used as a negative control. All tested strains were cultured overnight at 30°C. For the induced gene expression, 0.5 µg nisin/ml was added after 4 h. Next day, the OD₆₀₀ of each culture was measured and the cultures were diluted with fresh medium to equal densities. The diluted cultures were then centrifuged and sterilized through a

0.2 µm filter. Each supernatant, 200 µl, was added into the well (diam. 9 mm) in M17GST agar with a lawn of the indicator *L. monocytogenes* strain. The plate was incubated overnight at 30°C.

Results

Isolation of a nisin-resistant strain and *nsr* identification

Nisin resistant strains were screened from raw milk on M17GST agar plates with 5 µg nisin/ml. Eight colonies were picked onto a new plate containing 12.5 µg nisin/ml. One isolate, TML01, survived and was identified as *Lactococcus lactis*, according to 99% homology to 16S rRNA sequences of *L. lactis* strains (Supplementary Table 2). Genes for nisin production were not detected from *L. lactis* TML01 (data not shown).

Many nisin-resistant but non-producing strains express the NSR, a protease cleaving nisin between MeLan²⁸ and Ser²⁹ (Sun et al., 2009). To determine if the nisin-resistance of the strain TML01 was also caused by NSR, the *nsr* gene was sought by PCR with *nsr*-specific primers. The PCR showed that *L. lactis* TML01 contains an *nsr* gene. The gene was sequenced (accession number 1384934 in NCBI) and the derived amino acid sequence showed 99% homology to NSR nisin-resistance proteins, as shown in Supplementary Figure 1.

Construction of the food-grade vectors

The food-grade vector pLEB688 was constructed by replacing the *nisI* gene of pLEB590 with the novel *nsr* gene. New multiple cloning site were introduced by PCR using *nsr3* and *nsr4* primers (Fig. 1a). A transcription terminator from *Bifidobacterium longum* DJO10A *putA2* operon (Lee et al., 2008) was added downstream from *nsr* with the primer *nsr4*. For inducible gene expression, another food-grade vector pLEB689 was constructed by inserting the *nisZ* promoter with its own ribosome binding site and the initiation codon ATG into the *HindIII/BglII* sites of pLEB688 (Fig. 1b). Two nucleotides, AA, before the initiation codon ATG of *nisZ* were changed to CC, creating an *NcoI* restriction site for in-frame gene cloning. Lastly, the food-grade secretion vector was constructed by cloning the *usp45* signal sequence into pLEB689 by overlap PCR. Simultaneously, the *NcoI* was replaced with the *BspHI* site between the P_{*nisZ*} and the *SSusp45* (Fig. 1c). The last codon, GCT encoding Ala²⁷ in the *usp45* signal sequence, was changed to another alanine

codon GCC. Hence, a *NaeI* site was created, enabling blunt-end fusion with the *SSusp45* and a cloned gene with no extra *N*-terminal amino acids in the mature secreted protein (Fig. 1c).

Vector stability and nisin-resistance of *L. lactis* strains

The stabilities of the constructed food-grade vectors were examined with serial subcultures in non-selective M17GS media. All food-grade vectors were stably maintained for more than 100 generations. Only 2-4% of the cells carrying the food-grade vectors lost their nisin-resistance. Single colonies obtained from the M17GST+nisin agar plates were found to contain plasmids with the expected size and restriction pattern (data not shown).

Nisin-resistance levels of the food-grade vector strains were determined by culturing the strains with different nisin concentrations. Nisin-resistances of the NZ9000 strain harboring the different vectors are shown in Fig. 2. NZ9000 is a nisin-sensitive strain which could not grow even in the lowest nisin concentration tested, 1.25 µg/ml. All the other strains grew in all tested nisin concentrations but variations in growth were observed. The *nisI* strain was visibly suffering in 2.5 µg nisin/ml, and its growth was strongly retarded with 5 µg nisin/ml. Also, with 5 µg nisin/ml the strain carrying the secretion vector pLEB690 grew slower than the other *nsr* vector strains. Nevertheless, the novel food-grade vectors were shown to be stable and to provide higher nisin-resistance than the previously constructed nisin-selectable plasmids.

Intra- and extra-cellular protein production from the *nsr* food-grade vectors

Intracellular protein production from the expression vectors pLEB688 and pLEB689 was examined by the expression of the *Lactobacillus helveticus pepI* gene similarly as described by Takala and Saris (2002) (data not shown). To determine the applicability of the novel secretion vector pLEB690, a pediocin gene was fused with *SSusp45* in the vector. The *Lb. plantarum* pediocin gene *papA* without leader sequence was amplified by PCR with primers ped1 and ped2. The PCR product was digested by *Bgl*III and cloned into *NaeI*/*Bgl*III-cut pLEB690, resulting in the *papA* secretion plasmid pLEB691 (Fig. 1d). Plasmid pLEB691 was transferred into the non-pediocin-producer *L. lactis* NZ9000. Anti-listerial potential of the putative pediocin-secretion strain was tested by agar diffusion assay. *L. lactis* NZ9000 harboring the empty vector pLEB690 showed no anti-*Listeria* activity. *L. lactis* NZ9000 with the pediocin secretion plasmid pLEB691 exhibited antilisterial activity both in 0.5 µg nisin/ml - induced and non-induced conditions, indicating that the strain expressed and secreted pediocin from the plasmid (Fig 3). Nisin-induced pediocin production

was higher than that without induction. The native pediocin producer *Lb. plantarum* WHE 92 was still better *Listeria*-killer than the NZ9000/pLEB691 or the nisin producer *L. lactis* N8. However, the successful secretion of pediocin concluded that the food-grade vector pLEB690 can be regarded as a secretion vector for *L. lactis*.

Discussion

A nisin-resistance gene *nsr* was found in *L. lactis* TML01 isolated from raw milk. This new NSR showed 99% homology to previously reported nisin-resistance proteins derived from different *L. lactis* strains. The *nsr* gene is a good candidate as a food-grade selection marker due to its nisin inactivation capacity (Sun et al., 2009). Three food-grade vector variants, pLEB688, pLEB689 and pLEB690, were constructed with this new *nsr* gene as the selection marker (Fig. 1). All these vectors are small (< 3.8 kb) and stable (only 2 to 4% loss in 100 generations) for gene cloning.

The functionality of the *nsr* food-grade vectors for intracellular protein production was verified by cloning the *pepI* gene into the plasmids pLEB688 and pLEB689 followed by analysis of PepI activity (results not shown) similarly as described by Takala and Saris (2002). The potential for protein secretion of the vector pLEB690 was tested by cloning the lactobacillar pediocin gene *papA*. Pediocin is a broad-spectrum bacteriocin that shows particularly strong anti-*Listeria* activity (Dabour et al., 2009). Several studies have demonstrated that *L. lactis* is a good host for pediocin expression (Arques et al., 2008; Reviriego et al., 2007a; b). Nisin-inducible expression of pediocin has also been successfully applied (Renyé & Somkuti, 2009; Reviriego et al., 2007a). However, use of the *usp45* signal sequence has not been previously reported for pediocin secretion, and the secretion of pediocin from a food-grade vector with nisin selection is also novel. *L. lactis* NZ9000 carrying the pediocin plasmid, pLEB691, provided higher anti-listerial activity in presence of nisin than without nisin, showing that the *nisZ* promoter and nisin induction are functional in the pLEB690 vector. NSR is a protease reducing nisin activity and induction sensitivity (Liang et al., 2009; Sun et al., 2009), which could lead to inhibition of induction. However, the concentration of nisin needed for promoter induction is very low, compared to e.g. the concentrations used in food or for plasmid selection pressure. The recommended concentration for nisin induction is 0.1-5 ng/ml (Mierau & Kleerebezem, 2005). Here, the expression of the pediocin gene was induced with 500 ng nisin/ml, about 100-1000 times more than what is sufficient. It is unlikely that NSR would degrade nisin from 500 ng/ml to below the inducible concentrations before nisin induction would occur. The higher pediocin production with nisin showed that nisin induction occurred fast enough before NSR would degrade the nisin concentration below an inducible level.

Even though *L. lactis* is naturally resistant to pediocin, co-expression of the pediocin immunity gene with the structural gene has been shown to increase the pediocin production up to the wild-type level (Arques et al., 2008). Thus, in the wild-type, pediocin producer, *Lb. plantarum* WHE 92, the expression of the native immunity gene may explain why it was still clearly better pediocin producer than the heterologous host *L. lactis* with no immunity gene. However, in this work, the aim was not to produce large amounts of pediocin, but to show the applicability of pLEB690 by secreting biologically active pediocin. Moreover, *L. lactis* NZ9000 harboring pLEB691 could also produce pediocin without nisin induction due to the constitutive P45 promoter. The promoter P45 broadens the applicability of the vector, as it can be used in *L. lactis* strains lacking the nisin regulatory genes. To sum up, the food-grade secretion vector pLEB690 enables nisin selection in transformation, is small and stable, and it can be used both for constitutive and inducible secretion of polypeptides in *L. lactis*.

The *nsr* gene used in this study made cells more resistant to nisin than the gene *nisI*, which was used as a selection marker in previous food-grade vector pLEB590 (Fig. 2). In the previous study, plasmid pLEB590 enabled higher nisin concentration in selection than the *nsr* vector pVS40 (Takala & Saris, 2002; von Wright et al., 1990). This was probably because pVS40 (7.8 kb) is larger than pLEB590 (3.1 kb), which may have reduced the nisin-resistance capacity due to a copy number effect. The *nsr* plasmids in this study are smaller than 4 kb, and the expression of *nsr* is directed not only from its own promoter but also from P45 and P_{nisZ} promoters. These differences may lead to a stronger expression of *nsr*, explaining the obtained higher nisin-resistance than with previously constructed vectors.

However, the secretion vector pLEB690 grew slower than the other *nsr* vectors in higher nisin concentrations. This could be explained by the secretion of a 7-aa nonsense peptide, caused by the expression of a fusion with *SSusp45* and the 21-bp open frame in MCS after the *SSusp45* (Fig.1). In addition to the waste of energy, secretion of this useless peptide causes higher load for the sec-dependent secretion system, which may decelerate the growth. In the other *nsr* vector pLEB689, there is an in-frame stop codon 3 bp after the start ATG, thus saving energy from production of unwanted useless peptides.

With the *nisI* vector pLEB590, transformant colonies had to be selected by pour plate method (Takala & Saris, 2002). In this study, the *nsr* vectors could be selected by conventional surface spread plate method, when 1% v/v Tween 80 was added into the selection medium (results not shown). Tween 80 is a surfactant of which increases nisin solubility and reduces its adsorption onto surfaces (Mocquot & Lefebvre, 1956). However, the function of Tween 80 in nisin selection is not fully clear. After transformation, the cells spread onto the agar form a layer. By adsorbing nisin molecules, the cell layer may hinder nisin from the medium to reach the cells on the top of the layer. Tween 80 could

reduce nisin adsorption onto the dead cells, allowing nisin to diffuse through the cell layer to the live cells on the top. This would inhibit the growth of background colonies and allow transformant selection on the agar surface.

In conclusion, three food-grade vectors were constructed, all of which enabled higher nisin selection pressure than earlier published nisin selectable food-grade vectors. The secretion vector pLEB690 contains a novel combination of the *usp45* secretion signal, dual expression promoters (constitutive P45 and nisin-inducible P_{nisZ}), and food-grade nisin-resistance gene *nsr* selection marker. In particular, this vector could be useful for secreting bioactive peptides in the intestine or in food applications.

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Fig. 1 Vectors constructed in this work. P45: lactococcal constitutive promoter; P_{nisZ} : nisin-inducible promoter of *nisZ*; *SSusp45*: signal sequence of lactococcal gene *usp45*; *papA*: gene encoding mature pediocin; *nsr*: gene encoding the nisin-resistance protease; Ter: transcription terminator. Changed nucleotides are marked by gray shade. The functional ATG initiation codon of pLEB689 is bolded.

Fig. 2 Nisin-resistance analysis of *L. lactis* N8 (black square), *L. lactis* NZ9000 (empty diamond) and *L. lactis* NZ9000 strains containing pLEB688 (empty square), pLEB689 (black triangle), pLEB690 (empty triangle) and pLEB590 (black diamond). a, 0 µg nisin/ml; b, 1.25 µg nisin/ml; c, 2.5 µg nisin/ml; d, 5 µg nisin/ml.

Fig. 3 Agar diffusion bioassay with *Listeria monocytogenes* WSLC 1018 as indicator. A, 0.5 µg nisin/ml induced NZ9000/pLEB691; B, non-induced NZ9000/pLEB691; C, pediocin producer *Lb. plantarum* WHE 92; D, 0.5 µg nisin/ml induced NZ9000/pLEB690; E, non-induced NZ9000/pLEB690; F, nisin producer *L. lactis* N8.

Fig. 1

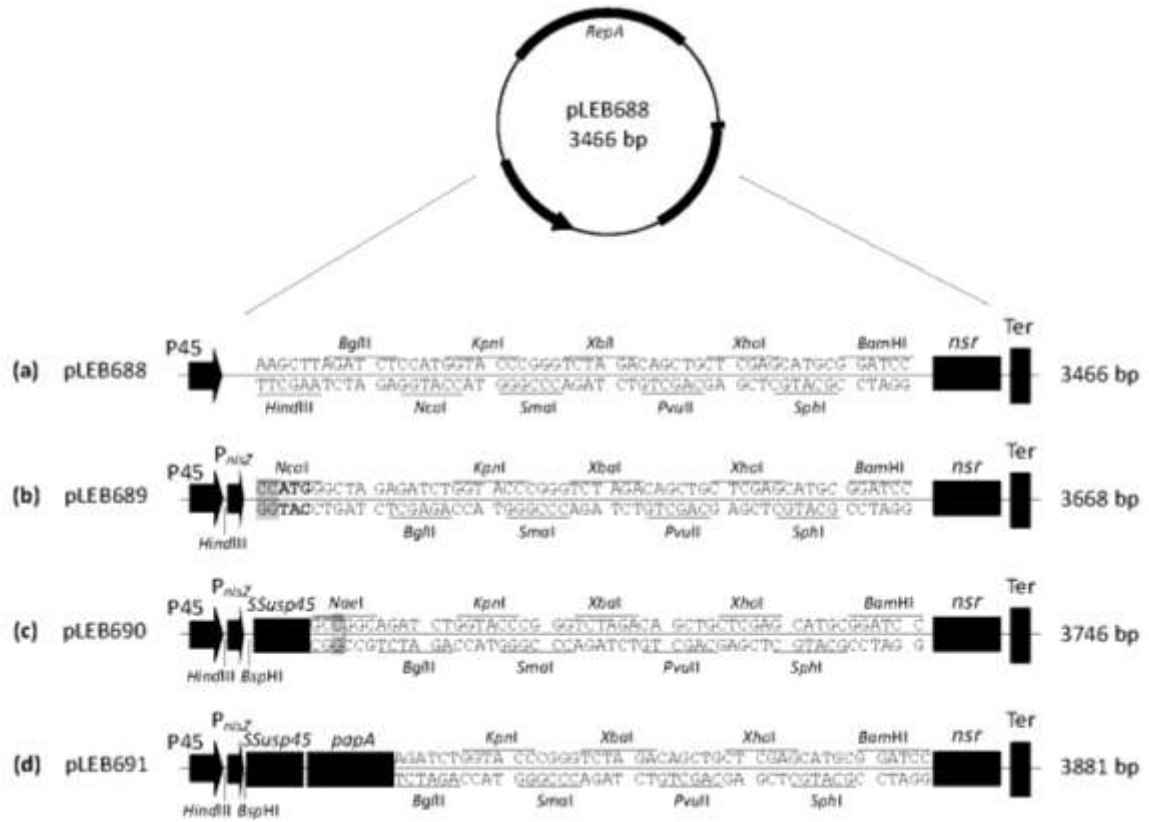


Fig. 2

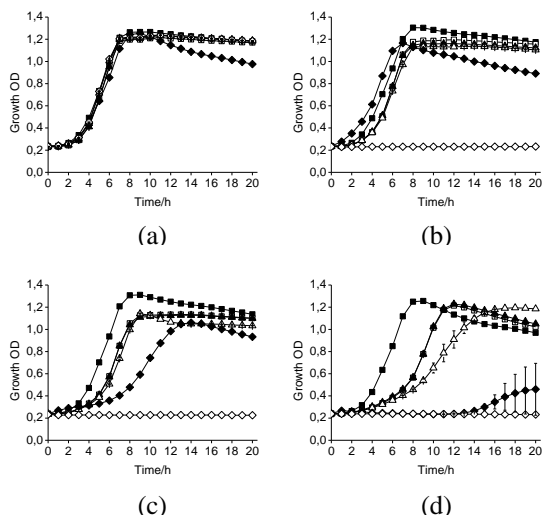
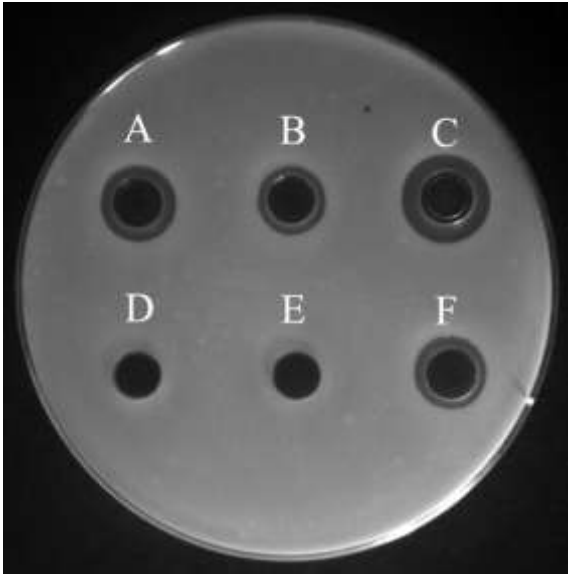


Fig. 3



Supplementary Table 1 Primers used in this study

Name	Sequence (5' to 3')	Restriction sites
16S1	TTTATTTGAGAGTTTGATCCTGG	
16S2	AAAGGAGGTGATCCAGCC	
nsr1	AAAACCTGCAGATAGCAATTCGGGTTT	
nsr2	CGGGATCCTTTCTTGGCAAGTCT	
nsr3	CCCAAGCTTAGATCTCCATGGTACCCGGGTCTAGACAGCTGCTCGAG CATGCGGATCCTTCCTAGATTTATCGTTTAC	<i>HindIII</i> , <i>BglII</i> , <i>NcoI</i> , <i>KpnI</i> , <i>SmaI</i> , <i>XbaI</i> , <i>PvuII</i> , <i>XhoI</i> , <i>SphI</i> , <i>BamHI</i>
nsr4	CCAGATATCACTAGTCATATGCAAAGGAGCCCGGCCCTGTAATGGG GTCGGGCTCCTTTGTCGACGTCCTTTTGCTTTACTTTAT	<i>EcoRV</i> , <i>SpeI</i> , <i>NdeI</i> , <i>SalI</i>
nsr5	GAAGATCTGGTACCCGGGTCTAGACAG	<i>BglII</i>
nsr6	TTTTCATGATGAGTGCCTCCTTATAATTTATTTTG	<i>BspHI</i>
PnisZ1	CCCAAGCTTAGTCTTATAACTATACTGACAATAGAAAC	<i>HindIII</i>
PnisZ2	GAAGATCTCTAGCCCATGGTGAGTGCCTCCTTATAATTTAT	<i>BglIII</i> , <i>NcoI</i>
usp1	CAAATAAATTATAAGGAGGCACTCATCATGAAAAAAAAAGATTATC TCAGC	<i>BspHI</i>
usp2	GAAGATCTGCCGGCGTAAACACCTGACAACG	<i>BglIII</i> , <i>NaeI</i>
ped1	AAATACTACGGTAATGGG	
ped2	GAAGATCTAGCATTATGATTACCTTGATG	<i>BglIII</i>

Supplementary Table 2 Homology of the 16S rRNA gene sequence of *Lactococcus lactis* TML01 with *L. lactis*

Accession	Description	Query coverage	Max ident
CP001834.1	<i>Lactococcus lactis</i> subsp. <i>lactis</i> KF147, complete genome	100%	100%
AE005176.1	<i>Lactococcus lactis</i> subsp. <i>lactis</i> II1403, complete genome	100%	100%
AM944595.1	<i>Lactococcus lactis</i> subsp. <i>cremoris</i> partial 16S rRNA gene, strain SC8	99%	99%
CP000425.1	<i>Lactococcus lactis</i> subsp. <i>cremoris</i> SK11, complete genome	100%	99%
AY675242.1	<i>Lactococcus lactis</i> strain SL3 16S ribosomal RNA gene, complete sequence	99%	99%
CP002094.1	<i>Lactococcus lactis</i> subsp. <i>cremoris</i> NZ9000, complete genome	100%	99%
AM406671.1	<i>Lactococcus lactis</i> subsp. <i>cremoris</i> MG1363, complete genome	100%	99%
DQ340069.1	<i>Lactococcus lactis</i> strain KLDS 4.0311 16S ribosomal RNA gene, partial sequence	97%	99%
AM157431.1	<i>Lactococcus lactis</i> 16S rRNA gene, clone 5C4	97%	99%
EU869288.1	<i>Lactococcus lactis</i> subsp. <i>lactis</i> strain 0711XYBLS 16S ribosomal RNA gene, partial sequence	97%	99%
HM004215.1	<i>Lactococcus lactis</i> subsp. <i>lactis</i> strain KLC02 16S ribosomal RNA gene, partial sequence	97%	99%
AB100800.1	<i>Lactococcus lactis</i> subsp. <i>lactis</i> bv. <i>diacetylactis</i> gene for 16S rRNA, partial sequence, strain: NIRD DRC-1	96%	100%

Supplementary Figure 1 Amino acid alignment of nisin-resistance proteins derived from different *L. lactis* strains

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A MKIGKRI LLGLVAVCALFLGI IYWG YKFN IYLVPPSPQKYVRVALKNMDELGLFTDSKEWVETKKKTI EETSNAKNYAETI PFLQKAIKVAGGKHSFIEHEEDIS 106
B MKIGKRI LLGLVAVCALFLGI IYWG YKFN IYLVPPSPQKYVRVALKNMDELGLFTDSKEWVETKKKTI EETSNAKNYAETI PFLQKAIKVAGGKHSFIEHEEDIS 106
C MKIGKRI LLGLVAVCALFLGI IYWG YKFN IYLVPPSPQKYVRVALKNMDELGLFTDSKEWVETKKKTI EETSNAKNYAETI PFLQKAIKVAGGKHSFIEHEEDIS 106
D MKIGKRI LLGLVAVCALFLGI IYWG YKFN IYLVPPSPQKYVRVALKNMDELGLFTDSKEWVETKKKTI EETSNAKNYAETI PFLQKAIKVAGGKHSFIEHEEDIS 106
E MKIGKRI LLGLVAVCALFLGI IYWG YKFN IYLVPPSPQKYVRVALKNMDELGLFTDSKEWVETKKKTI EETSNAKNYAETI PFLQKAIKVAGGKHSFIEHEEDIS 106

A KRSTIKYIKPKAEIEGNTLILTIPEFTGNDSQASDYANFLESSEHKNNYNGVIVDLRGNRGGDLSPMVLGLSPLL PDGTLFTYVDKSSSHSKFVELQNGEINSGGSS 212
B KRSTIKYIKPKAEIEGNTLILTIPEFTGNDSQASDYANFLESSEHKNNYNGVIVDLRGNRGGDLSPMVLGLSPLL PDGTLFTYVDKSSSHSKFVELQNGEINSGGSS 212
C KRSTIKYIKPKAEIEGNTLILTIPEFTGNDSQASDYANFLESSEHKNNYNGVIVDLRGNRGGDLSPMVLGLSPLL PDGTLFTYVDKSSSHSKFVELQNGEINSGGSS 212
D KRSTIKYIKPKAEIEGNTLILTIPEFTGNDSQASDYANFLESSEHKNNYNGVIVDLRGNRGGDLSPMVLGLSPLL PDGTLFTYVDKSSSHSKFVELQNGEINSGGSS 212
E KRSTIKYIKPKAEIEGNTLILTIPEFTGNDSQASDYANFLESSEHKNNYNGVIVDLRGNRGGDLSPMVLGLSPLL PDGTLFTYVDKSSSHSKFVELQNGEINSGGSS 212

A TRVSDNKKIKKAPIAVLIDNNTGSSGELTALCFEGIPNVKFLGSDSAGYTSANQTVYLYDGGSTLQITSAFVKDRITNNIYKNFPI SPDIQTNNAKSSAIEWIKSQIK 318
B TRVSDNKKIKKAPIAVLIDNNTGSSGELTALCFEGIPNVKFLGSDSAGYTSANQTVYLYDGGSTLQITSAFVKDRITNNIYKNFPI SPDIQTNNAKSSAIEWIKSQIK 318
C TRVSDNKKIKKAPIAVLIDNNTGSSGELTALCFEGIPNVKFLGSDSAGYTSANQTVYLYDGGSTLQITSAFVKDRITNNIYKNFPI SPDIQTNNAKSSAIEWIKSQIK 318
D TRVSDNKKIKKAPIAVLIDNNTGSSGELTALCFEGIPNVKFLGSDSAGYTSANQTVYLYDGGSTLQITSAFVKDRITNNIYKNFPI SPDIQTNNAKSSAIEWIKSQIK 318
E TRVSDNKKIKKAPIAVLIDNNTGSSGELTALCFEGIPNVKFLGSDSAGYTSANQTVYLYDGGSTLQITSAFVKDRITNNIYKNFPI SPDIQTNNAKSSAIEWIKSQIK 318

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Amino acid sequence: A, NSR of *L. lactis* TML01; B, nisin-resistance protein expressed by pND301 (Liu et al., 1996); C, nisin-resistance protein of *L. lactis* TS1640 (Sun et al., 2009); D, nisin-resistance protein of *L. lactis* subsp. *lactis* biovar *diacetylactis* DRC3 (Froeth and McKay, 1991); E, nisin-resistance protein expressed by pNP40 (O'Driscoll et al., 2006).

Froeth BR, McKay LL (1991) Molecular characterization of the nisin-resistance region of *Lactococcus lactis* subsp. *lactis* biovar *diacetylactis* DRC3. *Appl Environ Microbiol* 57:804-811

Liu CQ, Leelawatcharamas V, Harvey ML, Dunn NW (1996) Cloning vectors for lactococci based on a plasmid encoding resistance to cadmium. *Curr Microbiol* 33:35-39

O'Driscoll J, Glynn F, Fitzgerald GF, van Sinderen D (2006) Sequence analysis of the lactococcal plasmid pNP40: a mobile replicon for coping with environmental hazards. *J Bacteriol* 188:6629-6639

Sun Z, Zhong J, Liang X, Liu J, Chen X, Huan L (2009) Novel mechanism for nisin-resistance via proteolytic degradation of nisin by the nisin-resistance protein NSR. *Antimicrob Agents Chemother* 53:1964-1973