Genome shuffling of *Lactococcus lactis* subspecies *lactis* YF11 for improving nisin Z production and comparative analysis

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ABSTRACT

Nisin has been widely used in the food industry as a safe and natural preservative to increase the shelf time of many foods. In this study, genome shuffling was applied to improve nisin Z production of *Lactococcus lactis* ssp. *lactis* YF11 (YF11) via recursive protoplast fusion. Ultraviolet irradiation and diethyl sulfate mutagenesis were used to generate parental strains for genome shuffling. After 4 rounds of genome shuffling, the best-performing strain F44 was obtained, which showed dramatic improvements in tolerance to both glucose (ranging from 8 to 15% (wt/vol) and nisin (ranging from 5,000 to 14,000 IU/mL). Fed-batch fermentation showed that the nisin titer of F44 was up to 4,023 IU/mL, which was 2.4 times that of the starting strain YF11. Field emission scanning electron microscope micrographs of YF11 and F44 revealed the apparent differences in cell morphology. Whereas YF11 displayed long and thin cell morphology, F44 cells were short and thick and with a raised surface in the middle of the cell. With the increasing glucose and nisin content in the medium, cells of both YF11 and F44 tended to become shrunken; however, alterations in YF11 cells were more pronounced than those of F44 cells, especially when cultured in tolerance medium containing both nisin and glucose. Nuclear magnetic resonance analysis demonstrated that the structure of nisin from YF11 and F44 was the same. Expression profiling of nisin synthesis related genes by real-time quantitative PCR showed that the transcription level of nisin structural gene *nisZ* and immunity gene *nisI* of F44 was 48 and 130% higher than that of the starting strain YF11, respectively. These results could provide valuable insights into the molecular basis underlying the nisin overproduction mechanism in *L. lactis*, thus facilitating the future construction of industrial strains for nisin production.

Key words: genome shuffling, *Lactococcus lactis*, nisin

INTRODUCTION

Lactococcus lactis strains are commonly used as starter culture for milk-based fermentation products, such as cheese and yogurt. Certain strains of *L. lactis* are known to produce nisin, a cationic, polycyclic bacteriocin of 34 residues, including several unusual dehydro residues and thioether bridge lanthionines (Delves-Broughton et al., 1996). Nisin has a wide range of antimicrobial activity against gram-positive bacteria and also shows antimicrobial activity against gram-negative bacteria when in combination with a chelating agent, disodium EDTA (Hurst, 1981; Stevens et al., 1991). Nisin has been widely used in the food industry as a safe and natural preservative to increase the shelf time of a variety of foods, such as cheeses, canned vegetables, beverages, and various pasteurized dairy products. Moreover, the use of the antimastitic nisin-containing product Mast Out has been licensed by ImmuCell Corp. (Portland, ME) to Pfizer Animal Health (Cotter et al., 2005). Nisin has been used in the treatment of mastitis, a persistent inflammation of the udder, in lactating dairy cows (Cao et al., 2007).

Due to its wide range of commercial applications, many efforts have been made to increase nisin yield and cut production costs. Two strategies have been used to improve the cost-effectiveness of commercial production of nisin. One strategy is to optimize the fermentation process. For example, the influence of growth parameters on the fermentative production of nisin by *L. lactis* ssp. *lactis* A164 was investigated (Cheigh et al., 2002). Other strategies that have been used to increase nisin production are based on traditional (random mutation and selection) or gene engineering (overexpression of selected genes and intentional mutagenesis) methods (Kalra et al., 1973; Cheigh et al., 2005). Although the effectiveness of the above 2 approaches are well established, the classical strain breeding method is time intensive and laborious, and gene engineering is mainly based on the availability of the information of the genotype and phenotype correlations. Genome shuffling, a recently developed strategy for rapid metabolic engineering of production strains (Zhang et al., 2002), could overcome these limi-

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tations. One distinct advantage of genome shuffling is that strain improvement for complex phenotypes does not depend on the availability of genetic information of the target strain. Although this method is mainly based on protoplast fusion, it is different from protoplast fusion, as it allows for the genetic recombination between multiple parents at each generation and, thus, greatly increases the opportunity to obtain high-performance strains (Gong et al., 2009). Genome shuffling has been successfully used to improve the performance of a variety of strains. The initial application of this technology was to improve tylosin production from *Streptomyces fradiae*; 2 rounds of genome shuffling were sufficient to reach the efficiency that could be reached after 20 rounds of mutation and selection of classical strain breeding (Zhang et al., 2002). The success of genome shuffling motivated its wide application to development of high-yield strains for the production of various valuable bioproducts, such as lactic acid (Patnaik et al., 2002; Zheng et al., 2012), riboflavin (Chen et al., 2004), lipase (Lin et al., 2007), ethanol (Bajwa et al., 2010; Gao et al., 2012; Jingping et al., 2012), bioinsecticide (Jin et al., 2009), ayamycin (El-Gendy and El-Bondkly, 2011), avilamycin (Lv et al., 2013), and alkaliphilic lipase (Wang et al., 2012).

Nisin production is related to a strain's glucose and nisin tolerance. Glucose tolerance will lead to an increase in lactic acid concentration, which could provide more energy for nisin production. To prevent the influence of an increase in nisin production on the strain growth, high nisin tolerance is essential for a high nisin-producing strain. The objective of this study was to improve nisin production through simultaneously increasing glucose and nisin tolerance of a nisin-producing strain by genome shuffling using the parental strains derived from UV irradiation and diethyl sulfate (DES) mutagenesis. Fed-batch fermentation was done to provide comparative performance data of the shuffled strain and the starting strain. In addition, a preliminary investigation was conducted to explore the potential mechanisms underlying improved nisin production by microscopic analyses and real-time quantitative PCR (RT-qPCR).

MATERIALS AND METHODS

Microorganisms and Cultivation

The starting strain *L. lactis* ssp. *lactis* YF11 (YF11; under accession number CGMCC7.52) is deposited and preserved at the Center of Industrial Culture Collection (CICC, Beijing, China). *Micrococcus flavus* ATCC 10240, preserved in the laboratory, was used as an indicator strain for the bioassay of nisin.

All the *L. lactis* strains were preserved and cultured in seed medium (wt/vol) containing peptone (1.5%), yeast extract (1.5%), sucrose (2.0%), KH_2PO_4 (2.0%), NaCl (0.15%), corn steep liquor (0.3%), cysteine (0.26%), and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.015%). The pH value was adjusted to 7.2 before autoclaving at 121°C for 20 min. The tolerance agar plate was prepared similarly, except that agar (1.5%) was added and after autoclaving the autoclaved glucose and nisin were added. Luria-Bertani medium was used for culture of the indicator strain.

Nisin and Sucrose Activity Assay

The determination of nisin activity method was based on the plate diffusion method, which was first used by Tramer and Fowler (1964) and developed by Wolf and Gibbons (1996). *Micrococcus flavus* ATCC 10240 was used as the nisin-sensitive indicator strain. The standardized nisin concentrate (Kanyi, Tianjin, China) was used for nisin standards. A stock solution of nisin was prepared by mixing 0.1 g of nisin in 10 mL of 0.02 M HCl (10^6 IU/mL) and boiling for 5 min. The stock solution was diluted using 0.02 M HCl to standard nisin solutions. Five hundred microliters of fermentation broth was diluted with 500 μL of 0.02 M HCl. The mixture was then boiled for 5 min to remove cell-bound nisin by hot acid extraction and centrifuged at $2,152 \times g$ for 5 min at room temperature to remove the cells. The supernatant was appropriately diluted with 0.02 M HCl. After autoclaving, the 26-mL assay medium was cooled to about 45°C and then inoculated with 1% (vol/vol) indicator strain *M. flavus* ATCC 10240 buffer (the final concentration of the indicator strain was 10^7 cfu/mL). The medium was then poured into a sterile plate. The plate was placed at room temperature for 0.5 h to allow thorough solidification, and then was placed at 4°C for 24 h for precultivation (precultivation enhances nisin diffusion into the agar medium). Test wells were then bored into the assay agar plate (6 wells per plate) using a 7-mm-diameter glass tube to remove the agar from the well. Standard nisin solutions and test solutions were then put into individual wells (100 μL per well) and the plates were incubated at 30°C for 24 h. Zones of inhibition were measured. From the data a regression equation was calculated. Each assay of standard sample or the broth sample was performed in triplicate. The sucrose concentration in the broth was measured using HPLC (Hunt et al., 1977).

Construction of the Parental Strain Population for Genome Shuffling

Ultraviolet irradiation and DES mutagenesis were used for the preparation of parental strains from the

Figure 1. The process for generating higher nisin-producing *Lactococcus lactis* ssp. *lactis* mutants through genome shuffling.

starting strain YF11 (Figure 1). The cells were grown in a 100-mL flask containing 50 mL of seed medium at 30°C for 12 h and then subcultured to the seed medium containing 1.2% glycine for 6 h. After cultivation, the broth was centrifuged at $2,152 \times g$ for 5 min, the pellet was washed twice with sterile water and resuspended with sterile 0.01 M potassium phosphate buffer (pH 7.0), and the cell density was adjusted to about 1×10^6 cfu/mL. Then, 4 mL of the above cell suspension in a plate without cover was exposed to UV irradiation for 120 s at a distance of 30 cm from the UV lamp with a wavelength of 254 nm and power of 15 W (Philips), and the killing ratio was 82%. After appropriately diluting with sterile water, the suspension of survived cells was spread on the agar medium with glucose content ranging from 8 to 13% and a nisin content of 4,000 to 11,000 IU/mL. As Figure 1 shows, the strains grown on the nisin or glucose tolerant plates were then plated on nisin and glucose plates to select the nisin- and glucose-tolerant strain. The grown strains were replicated on the nisin assay agar medium plates inoculated with *M. flavus*

ATCC 10240. Strains with larger inhibition zones were selected. Selected candidates strains were subjected to fermentation test to identify strains with high nisin production. The process of DES mutagenesis was the same as that of UV irradiation, except the cell suspension was treated with 400 μ L of DES at 30°C for 35 min.

The mutants with subtle improvement were used as the initial strains for the second round of mutation according to the above procedure. The populations obtained from the second mutation selected for high content of glucose and nisin stress were used as the parental strains for genome shuffling.

Genome Shuffling

The parental strains were cultured at 30°C in 50 mL of seed broth for 12 h and then subcultured to seed broth (with 1.2% glycine) and incubated for 6 h at 30°C. Then, 2-mL cultures were harvested by centrifugation at $2,152 \times g$ for 5 min at room temperature and washed twice with *Lactococcus* protoplasting buffer (LPB),

which consisted of 10 mM Tris-HCl, 20 mM CaCl₂, and 0.5 M sucrose with pH 6.5. Then the precipitate was resuspended in 4 mL of LPB with lysozyme (2 mg/mL) and incubated at 37°C for 40 min in a rotary shaker with a speed of 100 rpm to enable complete digestion. The appearance of spherical cells was used as an indicator of protoplast formation. The efficiency of protoplast formation was calculated by serial dilutions on seed medium. Protoplasts were collected by centrifugation at $1,377 \times g$ for 8 min at 4°C and washed twice with 10 mL of LPB to remove the lysozyme before suspending in the same amount of LPB for further use.

Protoplasts of 500 µL of the different mutants were mixed, centrifuged at $1,377 \times g$ for 8 min at 4°C, and suspended in 100 µL of LPB. The suspension was divided into 2 parts and inactivated using 2 methods. One part was irradiated by UV irradiation for 120 s and the other part was incubated at 70°C for 60 min. The 2 parts were mixed together after inactivation and 900 µL of LPB containing 40% polyethylene glycol 4000 was added to the mixture, which was then incubated at 37°C for 6 min. Then, the fused protoplasts were centrifuged at $1,377 \times g$ for 8 min at 4°C, washed twice with 2 mL of LPB, and resuspended in 100 µL of LPB. The washed protoplasts were then poured into regeneration medium consisting of fermentation medium supplemented with 20 mM MgCl₂, 2.5% gelatin, 0.5 M sucrose, and 1.5% BSA, and then cultured at 30°C for 6 h for preregeneration. After preregeneration, the broth was serially diluted and then plated on different nisin and glucose plates. For the first round of genome shuffling, for example, different tolerance plates consisting of 12% and 11,000 IU/mL, 12% and 12,000 IU/mL, 12% and 13,000 IU/mL, 13% and 11,000 IU/mL, 13% and 12,000 IU/mL, and 13% and 13,000 IU/mL nisin and glucose concentrations, respectively, were used. Colonies that could grow in the medium with higher glucose and nisin content were named F1 and all the plates grown with strains were replicated on the nisin assay agar medium plates inoculated with *M. flavus* ATCC 10240 to select the strains with larger inhibition zones. Selected strains were subjected to a fermentation test to identify strains with high nisin production. The F1 strains with improved nisin production and glucose and nisin tolerance were selected for the next round of the shuffling process. Four rounds of genome shuffling were performed by repeating the process of protoplast fusion described above. The detailed procedure of genome shuffling is illustrated in Figure 1.

Assessment of Flask Fermentation

Colonies from seed plates were used to inoculate the seed medium for overnight cultivation. Fermentations

were conducted in 250-mL Erlenmeyer flasks containing 100 mL of fermentation medium inoculated with 5% of the overnight cultures and incubated for 16 h at 30°C with a slow agitation of 100 rpm to keep the fermentation broth homogeneous. Each strain was grown in 3 separate flasks. Samples were withdrawn every 2 h for cell density analysis, sugar concentration, and nisin production.

Fed-Batch Fermentation

After flask cultivation assessment, the fed-batch fermentation was conducted in a 3-L fermenter containing 2 L of initial fermentation medium [peptone (1.5%), yeast extract (1.5%), sucrose (1.0%), KH₂PO₃ (2.0%), NaCl (0.15%), corn steep liquor (0.3%), cysteine (0.26%), and MgSO₄·7H₂O (0.015%)]. The medium was inoculated with 100 mL of 10-h seed culture with an optical density at a wavelength of 600 nm (OD₆₀₀) of 1.5. The fermentations were conducted at 30°C with slow agitation (100 rpm) to keep the fermentation broth homogeneous. The initial pH of the fermentation broth was adjusted to 7.0 with 5 M NaOH. When naturally decreased to 6.2, the fermentation broth pH was maintained by automatically adding 5 M NaOH to the fermentation broth. A 500 g/L sucrose solution was added to the fermentation broth from 5 to 12 h at a rate of 20 mL/h. The fermentation broth was sampled every 2 h for cell density analysis, sugar concentration, and nisin production.

Nuclear Magnetic Resonance Analysis

The cultivation process of YF11 and F44 was the same as flask fermentation. And the purification process as Mulders et al. (1991) had done. The nuclear magnetic resonance (NMR) spectra were produced at room temperature 25°C in a Bruker DPX-300 instrument (Bruker Biosciences Corp., Billerica, MA). The dimethyl sulfoxide was used as the solvent.

Microscopic Analyses

Samples (5 mL) of fermentation broth (F44 and YF11 in different tolerance media incubated for 2 h at 30°C) were centrifuged at $2,152 \times g$ for 5 min at 4°C. The supernatant was discarded and the pellet was washed twice with 5 mL of 0.01 M potassium phosphate buffer (pH 7.0). The pellet was fixed with phosphate-glutaraldehyde (3% vol/wt) for 2 h, centrifuged at $2,152 \times g$ for 5 min at 4°C, and then washed twice with demineralized water. The concentrated cells were dehydrated with serial concentrations of ethanol (30, 50, 70, 90, and 100%, respectively) for 10 min. The cells

were dried with a vacuum freeze-drying instrument for 20 min and the resultant powder was coated with Pt and Pd for field emission scanning electron microscopy (FESEM) and examined with a Hitachi S-4800 field emission scanning electron microscope (Hitachi Ltd., Tokyo, Japan) operated at 1 kV.

RT-qPCR

To establish the quantitative ability of each RT-qPCR assay, a standard curve was generated. The genome of YF11 was used as the standard. The genome was diluted to concentrations of 10^{-5} , 10^{-4} , 10^{-3} , 10^{-2} , 10^{-1} , and 1 copy of DNA per μL . Two microliters of each dilution was combined with primers to make the standard curve of each target gene. And for the reference gene standard curve, the primers of 16S rRNA were added. For total RNA isolation, 1 mL of culture (mid-log cultures of F44 and YF11 incubated for 8 h at 30°C) was harvested by centrifugation at $2,152 \times g$ for 5 min at 4°C , and the pellet was used for RNA isolation with the ZR RNA MiniPrep (1064) kit from Zymo Research Corp. (The Epigenetics Co., Irvine, CA) according to the manufacturer's instructions. Complementary DNA was reverse transcribed from 5 μg of total RNA per reaction using the TIANScript RT Kit (Fermentas, New York, NY) according to the manufacturer's protocol. The RT-qPCR was performed on the Step One Plus Real-Time PCR system (Applied

Biosystems Inc., Foster City, CA), using Power SYBR Green PCR Master Mix (Applied Biosystems Inc.), according to the manufacturer's instructions. For the target gene, the cDNA was diluted 100 times and 2 μL of the dilution product was used as template; for the reference gene 16S rRNA, its cDNA was diluted 1,000 times. Quantitative PCR conditions were as follows: 1 cycle at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, and annealing and extension at 60°C for 1 min. The PCR products were analyzed for specificity and homogeneity by melting curve analysis. The primer sets used for quantitative PCR are listed in Table 1. The wild-type strain was used as the control.

RESULTS

Parental Strains Generated for Genome Shuffling

To generate parental strains for genome shuffling, starting strain YF11 was mutagenized by UV irradiation and DES, respectively. Parental strains with improvement in desired phenotypes, which reflect genetic diversity, are essential for successful genome shuffling (Figure 1). The nisin yield of strain YF11 was only 1,025 IU/mL in flask fermentation and its tolerance to glucose and nisin was 8% (wt/vol) and 5,000 IU/mL, respectively. Nisin resistance and glucose tolerance were used as selection markers. After 2 rounds of UV irradiation, 3 strains named U1, U2, and U3 with higher nisin and glucose tolerance were obtained. The

Table 1. Polymerase chain reaction primers used in this study

Gene name	Primer ¹	Primer sequence (5' to 3')	Primer size (bp)	Product size (bp)
<i>nisZ</i>	<i>nisZ_F1</i>	ACTTGGATTTGGTATCTGTTCG	23	114
	<i>nisZ_R1</i>	TCATGTTACAACCCATCAGAGC	23	
<i>nisB</i>	<i>nisB_F1</i>	CACAGGAAAACCCATTGGATA	21	147
	<i>nisB_R1</i>	CTACAACATCGGCAACTCTCC	21	
<i>nisT</i>	<i>nisT_F1</i>	TATCACAGCTTTTGTTCGGTTG	22	81
	<i>nisT_R1</i>	AGATGTCTCCCTGAACCTAGCA	22	
<i>nisC</i>	<i>nisC_F1</i>	GCGGGCTGTATCTTAGCTTATG	22	145
	<i>nisC_R1</i>	CTGCTACAAGTCCATCTTTCCA	22	
<i>nisI</i>	<i>nisI_F1</i>	GGCAAAAGTATCCCGAGGTC	20	86
	<i>nisI_R1</i>	CCATAATCCCATTCCGTCCT	20	
<i>nisP</i>	<i>nisP_F1</i>	AGAGAGAGCATTTTCCGTGAAG	22	182
	<i>nisP_R1</i>	TGACATAGATTTGGGGGTTAGG	22	
<i>nisR</i>	<i>nisR_F1</i>	TATTGCGTTCCTCTCGCTTTA	21	99
	<i>nisR_R1</i>	TATTGCGTTCCTCTCGCTTTA	21	
<i>nisK</i>	<i>nisK_F1</i>	CATTGAAGATGGGGGAAATAAC	22	163
	<i>nisK_R1</i>	GCAGCAACCTGGAAGGATAA	20	
<i>nisF</i>	<i>nisF_F1</i>	CAGGAAAGAAAAGAGCAGGAAA	21	141
	<i>nisF_R1</i>	ACAACCTCCGCAATACCATCAG	21	
<i>nisE</i>	<i>nisE_F1</i>	ATCAGAGGAGCAAGCTGGAA	20	156
	<i>nisE_R1</i>	CCTTGAACACAAAAGCAACC	21	
<i>nisG</i>	<i>nisG_F1</i>	CAGTAGGGCAACAAGGAATGA	21	198
	<i>nisG_R1</i>	CAGGTAAAGCAATAGGACACCA	21	
16S rRNA	16SrRNA_F1	AGCCTCAGTGTCTCAGTTACAG	20	148
	16SrRNA_R1	GTGGCTCAACCATTTGTATGC	20	

¹F1 = forward; R1 = reverse.

Figure 2. Comparison of the shuffled strains and the initial strain *Lactococcus lactis* ssp. *lactis* YF11 (YF11). (A) Comparison of glucose tolerance between the shuffled strains and the initial strain YF11; (B) comparison of the nisin tolerance between the shuffled strains and the initial strain YF11; (C) comparison of nisin production between the shuffled strains and the initial strain YF11; (D) comparison of the growth of strains in the different concentrations of glucose and nisin media.

glucose tolerance of U1, U2, and U3 was 10, 12, and 10%, respectively (Figure 2a), and the nisin tolerance of U1, U2, and U3 was 10,000, 9,000, and 9,000 IU/mL, respectively (Figure 2b). After 2 rounds of DES mutagenesis, the nisin and glucose tolerance of resultant mutants named H1, H2, and H3 increased from 8% and 5,000 IU/mL to 12% and 7,000 IU/mL (Figure 2a and b). Six strains, with nisin yields of 1,660, 1,720, 1,500, 1,596, 1,414, and 1,714 IU/mL were selected as the parental strains for genome shuffling.

Higher Nisin-Producing Recombinants Generated from Genome Shuffling

To conduct protoplast fusion-mediated genome shuffling, protoplasts of the 6 parental strains were prepared,

inactivated, mixed together, and then subjected to the first round of pool-wise recursive protoplast fusion. The resulting populations were screened for mutants capable of growing on plates containing higher levels of nisin and glucose, followed by evaluation of the ability of the selected mutants to produce nisin. Mutants with larger inhibition zones on the assay plates were selected for further confirmation in flask fermentation. Populations of the verified mutants were used for subsequent protoplast fusion.

After the first round of genome shuffling, 79 colonies were regenerated in the tolerance assay plate, and 10 strains exhibiting large inhibition zones on the replicate nisin assay plates were selected for quantitative assessment of nisin production. Four strains named F11, F12, F13, and F110 produced higher nisin yields (2,245,

2,267, 1,857, and 1,972 IU/mL, respectively), compared with the 6 parental strains (Figure 2c). These 4 strains were used for protoplast formation and subjected to the second round of genome shuffling. Four isolates named F21, F22, F23, and F24 were selected from 86 regenerated colonies on selective plates, and their nisin production was 2,337, 2515, 2,530, and 2,870 IU/mL, respectively (Figure 2c). The selected 4 mutants were subjected to the third round of genome shuffling, and 3 fusion mutants named F34, F35, and F37 were selected from 82 regenerated colonies. The nisin production of the 3 shuffled strains was 2,661, 2,892, and 2,931 IU/mL, respectively (Figure 2c). Then, the fourth round of genome shuffling was conducted using the above 3 isolates. Three strains named F42, F43, and F44 were identified from the resulting population (93 colonies) of the fourth-round protoplast fusion. Their nisin titers were 3,100, 3,457, and 3,650 IU/mL, respectively (Figure 2c). After 4 rounds of protoplast fusion, no significant increases in the tolerance levels to glucose and nisin were observed. Nisin production yields were observed for the obtained fusion mutants (data not shown), and the best-performing shuffled strain F44 was chosen for further study.

The tolerance of these selected shuffled strains was investigated using media containing different glucose and nisin concentrations. The results are shown in Figure 2d; compared with the wild strain YF11, the counts (cfu/mL) of the shuffled strains tended to be lower, but the changes were not significant.

Fermentation Performance of the Shuffled Strains

To characterize nisin production performance among the selected mutant strains, shake-flask fermentation was performed. Cell density and nisin production were monitored. Strains F44, F37, F24, and the starting strain YF11 were grown on agar plates containing 14,000 IU of nisin/ml and 14% (wt/vol) glucose. As shown in Figure 3a, all the shuffled strains grew to lower optical densities than the starting strain under the conditions assayed. As shown in Figure 3b, nisin production started to increase in parallel to the growth of strains. Nisin production of the shuffled strain F44 was significantly higher than that of the starting strain YF11. Figure 3c shows an obvious difference between F44 and another 3 strains (YF11, F24, and F37). For F44, the sucrose concentration decreased sharply in the beginning 6 h; after 6 h, the consumption tended to be slow and the sucrose concentrations became stable after 12 h. However, the sucrose consumption of the other strains during the first 4 h was slow compared with F44 and began to become faster during the following 2 h.

The fed-batch fermentation was supplemented with sucrose and maintained at constant pH (6.2) to further increase nisin production of F44. As shown in Figure 4a, YF11 grew faster initially, and reached the steady phase after 8 h of fermentation, whereas F44 reached steady phase after 14 h of fermentation. The optical density value of F44 was higher than that of YF11. The pH of the fermentation broth of both YF11 and F44 reached 6.2 after 6 h fermentation (Figure 4b).

Figure 4c shows that the difference of nisin production between YF11 and F44 was not apparent during the first 4 h of fermentation. However, the nisin production rate of F44 accelerated after 4 h and was higher than that of YF11. Strain F44 reached its nisin production peak of 4,023 IU/mL at 14 h, whereas after 12 h of fermentation YF11 reached its highest nisin titer of 1,676 IU/mL. Another important feature was that after F44 reached its peak, its nisin titer in the fermentation broth displayed a rapid decline, whereas strain YF11 showed no such trend. The reason for rapid decreases was unclear. It is interesting that a similar phenomenon was also observed for natamycin production (Luo et al., 2012).

Figure 4d shows the difference in sucrose consumption between YF11 and F44. The results were somehow different with the flask fermentations. At the beginning, YF11 consumed more sugars; however, after about 6 h, the consumption of sucrose of F44 exceeded that of YF11. It might be very useful to investigate the by-product of YF11 and F44.

Characterization of the Shuffled Strain F44

The genetic stability of F44 was investigated by 6 successive cultivations. Its nisin yield ranged from 4,019 to 4,028 IU/mL, suggesting that the shuffled strain F44 was genetically stable.

Given that F44 exhibited a different colony morphology compared with the initial strain YF11 (Figure 5a), FESEM was applied to further investigate the morphological changes. Strains YF11 and F44 were cultivated in the medium with different concentrations of glucose and nisin for 2 h and the cells prepared as powder for FESEM analysis. As shown in Figure 5b, FESEM micrographs of YF11 and F44 revealed the apparent differences in cell shape. Whereas YF11 displayed a long and thin cell shape, F44 cells were short and thick and with a raised surface in the middle of the cell.

In this work, the relationship between nisin and glucose tolerance and nisin production of the nisin-producing strain was investigated using nisin and glucose as the selection markers. Strains that cannot tolerate the levels of nisin and glucose in the medium broke up after 2 h cultivation. As shown in Figure 6, a mor-

Figure 3. Characterization of the *Lactococcus lactis* ssp. *lactis* strains by the shake-flask fermentation process. (A) Optical density at a wavelength of 600 nm (OD₆₀₀); (B) nisin production; (C) sucrose consumption. Average data of triplicate experiments are presented; error bars represent the standard deviation of triplicate experiments.

phological comparison between the initial strain YF11 and the highest nisin-producing strain F44 revealed that with increasing glucose and nisin content in the medium, cells of both YF11 and F44 tended to become shrunk; however, alterations in YF11 cells were more pronounced than those of F44 cells, especially when cultured in tolerance medium containing both nisin and glucose.

Expression Profiling of Nisin Production-Associated Genes in the Shuffled Strain F44

To investigate the potential mechanism of higher levels of nisin production in mutant F44, RT-qPCR was used to probe the expression levels of the nisin biosynthesis-related genes between strains F44 and YF11. Eleven genes related to nisin production were examined.

The mid-log phase cells at mid-log phase (8 h) was harvested for gene expression. As shown in Figure 4a, we determined that the rate of cell growth was almost the same and, in theory, at this period, the expression of the genes should be the highest.

The genome of YF11 was used as the standard for standard curve generation. The expression was measured in high-producing strain F44 and wild-type YF11.16S rRNA was used as the reference gene. The F44 gene expression relative to YF11 was calculated using the following formula and the gene expression of wild-type YF11 was considered 1:

Relative gene expression in F44 =

$$\frac{\text{gene in F44 expression}/16\text{S RNA in F44 expression}}{\text{gene in YF11 expression}/16\text{S RNA in YF11 expression}}$$

As shown in Figure 7, the expression level of structural gene *nisZ* encoding the nisin Z precursor of F44 was 48% higher than that of the initial strain YF11. The expression levels of genes involved in the post-translational modification process were all significantly enhanced. Further, the expression level of nisin immunity gene *nisI* of strain F44 was 1.3 times higher than that of the initial strain YF11. However, the expression levels of other nisin immunity-related genes *nisEFG* of F44 were lower than that of YF11. These results could provide valuable insights into the molecular basis of nisin overproduction in *L. lactis*, thus facilitating future engineering of further improved industrial strains for nisin production.

NMR Analysis

Genome shuffling is a technology for engineering phenotypes at the whole genome level. It offers the ad-

Figure 4. Fed-batch fermentation of *Lactococcus lactis* ssp. *lactis* YF11 and F44. (A) Optical density at a wavelength of 600 nm (OD_{600}); (B) pH; (C) nisin production; (D) sucrose consumption. Average data of triplicate experiments are presented; error bars represent the standard deviation of triplicate experiments.

vantage of simultaneous changes at different positions throughout the entire genome. As for the increase in nisin activity in the shuffled strain F44, the conclusion that the nisin production increase was not convincing may be due to the change in nisin structure. Therefore, the structure of the peptide purified from strains YF11 and F44 was investigated by NMR techniques and compared. From Figure 8, it can be seen that the sequential assignments of the 2 spectra were the same and the same as the published nisin Z structure (Mulders et al., 1991). Thus, we could make the conclusion that the nisin activity increase in the F44 fermentation broth was due to the increase in nisin production.

DISCUSSION

Engineering microbial cells for the production of industrially valuable compounds is a major objective

of biotechnological research. It has been reported that both substrate and product inhibition play an important role for an efficient fermentation process (Åkerberg et al., 1998; Jia et al., 2006). Yu et al. (2007) reported that the increase of glucose tolerance of *L. lactis* could increase lactic acid production. The nisin production process has a growth-dependent pattern; therefore, nisin yield could be increased with increasing glucose tolerance of *L. lactis*. Meanwhile, increasing nisin tolerance could also enhance nisin yield by the producing organism (Qiao et al., 1997). Thus, the strain with simultaneous high glucose and nisin tolerance could result in considerable economic benefits for industrial applications. Genome shuffling, a technology for strain improvement based on protoplast fusion, has been successfully used to increase substrate and product tolerance of microbes (Wang et al., 2007; John et al., 2008; Li et al., 2012). Unlike the rational methods for im-

Figure 5. Micrographs of *Lactococcus lactis* ssp. *lactis* YF11 and F44. (A) Petri culture dish graphs; (B) field emission scanning electron microscopy micrographs of YF11 and F44 cultured in the tolerance medium. The YF11 micrographs are shown in subpanels a to c and F44 micrographs are shown in subpanels d to f. Color version available in the online PDF.

proving microbial strains, genome shuffling could cause simultaneous changes genome wide efficiently without the limitation of microbes of clear genetic background (Gong et al., 2009). Moreover, the efficiency for generating phenotypic improvement is higher compared with the classical strain breeding method. Thus, in the current study, genome shuffling was used to improve nisin production of *L. lactis* through simultaneously increasing glucose and nisin tolerance of the strain.

After 4 rounds of genome shuffling, a high nisin-producing strain F44 was constructed. Six successive subcultures confirmed the genetic stability of F44. Fed-

batch fermentation results showed that the nisin yield of F44 was 2.4 times that of the starting strain YF11. These results demonstrate that it is efficient to improve nisin yield through simultaneously improving nisin and glucose tolerance by genome shuffling.

Genome shuffling is a technology for engineering phenotypes at the whole genome level. It offers the advantage of simultaneous changes at different positions throughout the entire genome. The genetic factors that confer the desired phenotype can be determined through the analysis and evaluation of the constructed strain. Therefore, NMR was conducted to study wheth-

Figure 6. Field emission scanning electron microscopy micrographs of *Lactococcus lactis* ssp. *lactis* YF11 and F44. Panels a to c and d to f represent the cell shape cultured in the glucose medium (8, 12, and 14% content, respectively) of YF11 and F44; panels h to j and k to m represent the cell shape cultured in the nisin medium (4,000, 12,000, and 14,000 IU/mL content, respectively) of YF11 and F44; panels m to o and p to r represent the cell shape cultured in the glucose and nisin medium (8% + 4,000 IU/mL, 12% + 8,000 IU/mL, 14% + 14,000 IU/mL content, respectively) of YF11 and F44.

Figure 7. Relative expression levels of key genes as affected by genome shuffling through real-time quantitative PCR in *Lactococcus lactis* ssp. *lactis* F44 compared with the initial strain YF11. Average data of triplicate experiments are presented; error bars represent the standard deviation of triplicate experiments.

er the structure of nisin had changed and RT-qPCR was conducted to investigate the transcription of nisin synthesis genes of the recombinant strain F44 and the starting strain YF11. The sequential assignment of the 2 spectra of nisin from YF11 and F44 was the same. The conclusion that the nisin structure was not changed though genome shuffling could be made.

The genes related to nisin synthesis, maturation, tolerance, and regulation are located on a conjugative transposon, Tn5276 (Dodd et al., 1990). Comparative analysis by RT-qPCR showed that the transcript levels for the structural gene *nisZ* in mutant F44 was 48% greater than that of the initial strain YF11, suggesting the relevance of high levels of *nisZ* expression for nisin overproduction. The unmodified precursor of nisin is processed by a specific modification process, including nisin maturation by NisB dehydratase (Koponen et al., 2002; Kuipers et al., 2006), cyclization mediated by enzyme NisC (Li et al., 2006), transportation by NisT (Qiao and Saris, 1996; Kuipers et al., 2004), and cleavage of the N-terminal leader sequence by protease NisP (Kuipers et al., 1993), eventually leading to the release of the biologically active nisin. We also provided evidence that all these posttranslational modification genes were upregulated, to various degrees, in F44 compared with the initial strain YF11 (Figure 7).

Nisin tolerance is conferred by 2 different systems: the lipoprotein NisI and ABC transporter NisFEG (Siegers and Entian, 1995); NisI and NisFEG are encoded by *nisI* and *nisFEG*. The expression level of *nisI* of F44 was 1.3 times that of the initial strain YF11, whereas the *nisFEG* expression level of F44 was lower than YF11. It has been documented that NisI enhanced

Figure 8. The nuclear magnetic resonance analysis of nisin produced by *Lactococcus lactis* ssp. *lactis* YF11 and F44.

nisin immunity of *L. lactis* in the strain expressing *nisFEG* compared with the strains lacking these genes (Takala et al., 2004). Therefore, it might be interesting to test whether further improvement in nisin tolerance through overexpression of *nisFEG* genes in F44 will lead to the construction of a recombinant strain with even higher nisin production.

CONCLUSIONS

Genome shuffling was shown to be applicable to greatly improve the nisin and glucose tolerance and nisin production of the starting strain YF11. Six strains with subtle improvements in glucose and nisin tolerance obtained by UV irradiation and DES mutagenesis were used to develop a higher nisin-producing mutant strain F44 after 4 rounds of genome shuffling based on screening for fusion mutants with high nisin and glucose tolerance. The fed-batch fermentation showed that the nisin production of F44 was 4023 IU/mL, whereas that of YF11 was 1676 IU/mL. The FESEM study showed strain F44 exhibited an apparent alteration in cell morphology, characterized by short, coarse, and thick appearance, and with a raised surface in the middle of the cell compared with the initial strain YF11. Expression profiling of nisin synthetic genes by RT-qPCR revealed their differential expression levels between the initial strain YF11 and the shuffled strain F44. These results could provide valuable clues to the future construction of better nisin production industrial strains of *L. lactis*.

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